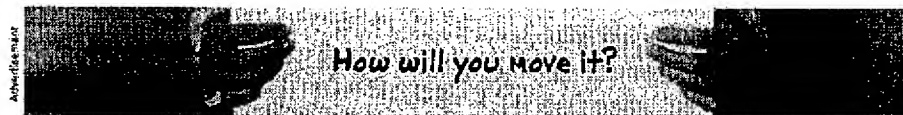


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EFFECTS OF CLOPIDOGREL IN ADDITION TO ASPIRIN IN PATIENTS WITH ACUTE CORONARY SYNDROMES WITHOUT ST-SEGMENT ELEVATION

THE CLOPIDOGREL IN UNSTABLE ANGINA TO PREVENT RECURRENT EVENTS TRIAL INVESTIGATORS*

ABSTRACT

Background Despite current treatments, patients who have acute coronary syndromes without ST-segment elevation have high rates of major vascular events. We evaluated the efficacy and safety of the antiplatelet agent clopidogrel when given with aspirin in such patients.

Methods We randomly assigned 12,562 patients who had presented within 24 hours after the onset of symptoms to receive clopidogrel (300 mg immediately, followed by 75 mg once daily) (6259 patients) or placebo (6303 patients) in addition to aspirin for 3 to 12 months.

Results The first primary outcome — a composite of death from cardiovascular causes, nonfatal myocardial infarction, or stroke — occurred in 9.3 percent of the patients in the clopidogrel group and 11.4 percent of the patients in the placebo group (relative risk with clopidogrel as compared with placebo, 0.80; 95 percent confidence interval, 0.72 to 0.90; $P < 0.001$). The second primary outcome — the first primary outcome or refractory ischemia — occurred in 16.5 percent of the patients in the clopidogrel group and 18.8 percent of the patients in the placebo group (relative risk, 0.86, $P < 0.001$). The percentages of patients with in-hospital refractory or severe ischemia, heart failure, and revascularization procedures were also significantly lower with clopidogrel. There were significantly more patients with major bleeding in the clopidogrel group than in the placebo group (3.7 percent vs. 2.7 percent; relative risk, 1.38; $P = 0.001$), but there were not significantly more patients with episodes of life-threatening bleeding (2.1 percent vs. 1.8 percent, $P = 0.13$) or hemorrhagic strokes.

Conclusions The antiplatelet agent clopidogrel has beneficial effects in patients with acute coronary syndromes without ST-segment elevation. However, the risk of major bleeding is increased among patients treated with clopidogrel. (N Engl J Med 2001;345:494-502.)

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THROMBOSIS caused by a ruptured or eroded atherosclerotic plaque is the usual underlying mechanism of acute coronary syndromes.¹ Aspirin and heparin reduce the risk of death from cardiovascular causes, new myocardial infarction, and recurrent ischemia,^{2,3} but there is still a substantial risk of such events in both the short term and the long term. Intravenous glycoprotein IIb/IIIa receptor blockers have been shown to reduce the incidence of early events, mainly among patients

who are treated according to an invasive strategy,^{4,5} but long-term oral therapy with glycoprotein IIb/IIIa receptor blockers is not beneficial and may even increase mortality.⁶ Similarly, continuing treatment with low-molecular-weight heparin beyond one week has not been shown to be effective.⁷ Although the long-term use of oral anticoagulants may be useful, no convincing evidence of their benefit is yet available.⁸ Therefore, there is a need to reduce further the risk of ischemic events in a broad spectrum of patients both when they first present with acute coronary syndromes and in the long term.

The thienopyridine derivatives, ticlopidine and clopidogrel, are antiplatelet agents that inhibit the platelet aggregation induced by adenosine diphosphate, thereby reducing ischemic events.⁹ Combining one of these drugs with aspirin, which blocks the thromboxane-mediated pathway, may have an additive effect. In patients who are undergoing percutaneous transluminal coronary angioplasty (PTCA) with stenting, short-term aspirin treatment plus a thienopyridine derivative results in a substantially lower rate of myocardial infarction than does either aspirin alone or warfarin.¹⁰ However, the role of long-term combined therapy with aspirin and an antiplatelet agent in a broader group of patients at high risk for cardiovascular events is unknown. We therefore designed the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial to compare the efficacy and safety of the early and long-term use of clopidogrel plus aspirin with those of aspirin alone in patients with acute coronary syndromes and no ST-segment elevation.

METHODS

Study Design

We undertook a randomized, double-blind, placebo-controlled trial comparing clopidogrel with placebo in patients who presented with acute coronary syndromes without ST-segment elevation. The design and rationale of the study have been reported previously.⁹

Study Patients

Patients were eligible for the study if they had been hospitalized within 24 hours after the onset of symptoms and did not have ST-segment elevation. Initially, patients older than 60 years

The Manuscript Writing Committee (Salim Yusuf, D.Phil., F.R.C.P.C., Feng Zhao, M.Sc., Shamir R. Mehta, M.D., F.R.C.P.C., Susan Chrolavicius, B.Sc., Gianni Tognoni, M.D., and Keith K. Fox, M.D., F.R.C.P.) assumes responsibility for the overall content of the manuscript. Address reprint requests to Dr. Yusuf at the Canadian Cardiovascular Collaboration Project Office, Population Health Research Institute, McMaster University, Hamilton General Hospital, 237 Barton St. E., Hamilton, ON L8L 2X2, Canada, or at yusufs@mcmaster.ca.

*The Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial investigators are listed in the Appendix.

of age with no new electrocardiographic changes but with a history of coronary artery disease were included. However, after a review of the overall rates of events among the first 3000 patients, the steering committee recommended that we enroll only patients who had either electrocardiographic changes or an elevation in the serum level of cardiac enzymes or markers at entry. We excluded patients with contraindications to antithrombotic or antiplatelet therapy, those who were at high risk for bleeding or severe heart failure, those who were taking oral anticoagulants, and those who had undergone coronary revascularization in the previous three months or had received intravenous glycoprotein IIb/IIIa receptor inhibitors in the previous three days.

After we had obtained written informed consent, patients were randomly assigned to either the clopidogrel group or the placebo group by a central, 24-hour, computerized randomization service. Permuted-block randomization, stratified according to clinical center, was used. A loading dose of clopidogrel (300 mg orally) or matching placebo was administered immediately, followed by clopidogrel (75 mg per day) or matching placebo for 3 to 12 months (mean duration of treatment, 9 months). Aspirin (recommended dose, 75 to 325 mg daily) was started or continued simultaneously with the study drug. Follow-up assessments occurred at discharge, at one and three months, and then every three months until the end of the study.

Study Organization

Patients were recruited between December 1998 and September 2000 at 482 centers in 28 countries. The ethics review board at each institution approved the study. The study was organized and coordinated and all the data were managed and analyzed by the Canadian Cardiovascular Collaboration Project Office, McMaster University, Hamilton, Ontario. A steering committee consisting of national coordinators oversaw the study. The data were periodically reviewed by an independent data and safety monitoring board.

Outcomes

The first primary outcome was the composite of death from cardiovascular causes, nonfatal myocardial infarction, or stroke, and the second primary outcome was the composite of the first primary outcome or refractory ischemia. The secondary outcomes were severe ischemia, heart failure, and the need for revascularization. The safety-related outcomes were bleeding complications, which were categorized as life-threatening, major (requiring the transfusion of 2 or more units of blood), or minor. All primary outcomes and life-threatening and major bleeding complications were adjudicated by persons who were unaware of the patients' treatment-group assignments.

Definitions

Death from cardiovascular causes was defined as any death for which there was no clearly documented nonvascular cause. Myocardial infarction was defined by the presence of at least two of the following: ischemic chest pain; the elevation of the serum levels of cardiac markers or enzymes (troponin, creatine kinase, creatine kinase MB isoenzyme, or other cardiac enzymes) to at least twice the upper limit of the normal reference range or three times the upper limit of normal within 48 hours after percutaneous coronary intervention (or to a level 20 percent higher than the previous value if the level had already been elevated because of an early myocardial infarction); and electrocardiographic changes compatible with infarction.⁹ Stroke was defined as a new focal neurologic deficit of vascular origin lasting more than 24 hours. Stroke was further classified as the result of intracranial hemorrhage, ischemia (if a computed tomographic or magnetic resonance imaging scan was available), or uncertain cause.

Refractory ischemia in the hospital was defined as recurrent chest pain lasting more than five minutes with new ischemic electrocardiographic changes while the patient was receiving optimal medical therapy (two antianginal agents, one of which was intravenous nitrate unless such therapy was contraindicated) and leading to ad-

ditional interventions (such as thrombolytic therapy, cardiac catheterization, the insertion of an intraaortic balloon pump, coronary revascularization, or transfer to a referral hospital for an invasive procedure) by midnight of the next calendar day. Refractory ischemia after discharge was defined by rehospitalization lasting at least 24 hours for unstable angina, with ischemic electrocardiographic changes. Severe ischemia (in the hospital) was defined as ischemia that was similar to in-hospital refractory ischemia but for which no urgent intervention was performed. Recurrent angina (in the hospital) was defined similarly, but electrocardiographic changes were not required.

Major bleeding episodes were defined as substantially disabling bleeding, intraocular bleeding leading to the loss of vision, or bleeding necessitating the transfusion of at least 2 units of blood. Major bleeding was classified as life-threatening if the bleeding episode was fatal or led to a reduction in the hemoglobin level of at least 5 g per deciliter or to substantial hypotension requiring the use of intravenous inotropic agents, if it necessitated a surgical intervention, if it was a symptomatic intracranial hemorrhage, or if it necessitated the transfusion of 4 or more units of blood. Minor bleeding episodes included other hemorrhages that led to the interruption of the study medication.

Statistical Analysis

The study was initially designed to include 9000 patients, with an expected rate of events in the placebo group of 12 to 14 percent. However, because the rate of events appeared to be lower than had originally been expected, the size of the study was increased. Assuming a rate of 10 percent in the placebo group for the first primary outcome and a two-sided alpha level of 0.045, a study with 12,500 patients would have 90 percent power to detect a 16.9 percent reduction in risk. For the second primary outcome, assuming a 14 percent rate of events in the placebo group and a two-sided alpha level of 0.01, the study had 90 percent power to detect a reduction of 16.4 percent in risk. Partitioning the alpha maintains an overall level of 0.05, after adjustment for the overlap between the two sets of outcomes. All analyses were based on the intention-to-treat principle and used either the log-rank statistic or the chi-square test. Subgroup analyses were conducted with the use of tests for interactions in the Cox regression model.

The data and safety monitoring board monitored the incidence of the primary outcome to determine the benefit of clopidogrel, using a modified Haybittle-Peto boundary of 4 SD in the first half of the study and 3 SD in the second half of the study. The boundary had to be exceeded at two or more consecutive time points, at least three months apart, for the board to consider terminating the study early. There were two formal interim assessments performed at the times when approximately one third and two thirds of the expected events had occurred. Despite the fact that the preset boundary indicating efficacy had been crossed by the time of the second interim analysis, the board recommended that the trial continue until its planned end, in order to define more clearly whether the risks of major bleeding episodes could offset the benefits of therapy.

All unrefuted events that occurred up to the end of the scheduled follow-up period on December 6, 2000, are included in the analyses. Vital status was ascertained for 12,549 of the 12,562 patients who underwent randomization (99.9 percent), with 6 patients in the clopidogrel group and 7 in the placebo group lost to follow-up.

RESULTS

The base-line characteristics of the patients are shown in Table 1.

Primary Outcomes

The first primary outcome — death from cardiovascular causes, nonfatal myocardial infarction, or stroke — occurred in 582 of the 6259 patients in the clo-

pidogrel group (9.3 percent) as compared with 719 of the 6303 patients in the placebo group (11.4 percent); relative risk, 0.80; 95 percent confidence interval, 0.72 to 0.90; $P < 0.001$) (Fig. 1 and 2 and Table 2). The rate of the second primary outcome — death from cardiovascular causes, nonfatal myocardial infarction, stroke, or refractory ischemia — was also higher in the placebo group (1187 patients [18.8 percent]) than in the clopidogrel group (1035 patients [16.5 percent]; relative risk, 0.86; 95 percent confidence interval, 0.79 to 0.94; $P < 0.001$). The rate of each component of these composite outcomes also tended to be lower in the clopidogrel group. However, the clearest difference was observed in the rates of myocardial infarction (Table 2). With respect to refractory ischemia, the difference was observed primarily in first events that occurred during the initial hospitalization (85 in the clopidogrel group as compared with 126 in the placebo group; relative risk, 0.68; 95 percent confidence interval, 0.52 to 0.90; $P = 0.007$), with little difference in the rate of rehospitalization for unstable angina.

Other In-Hospital Outcomes

Significantly fewer patients in the clopidogrel group than in the placebo group had severe ischemia (176 patients [2.8 percent] vs. 237 patients [3.8 percent]; relative risk, 0.74; 95 percent confidence interval, 0.61 to 0.90; $P = 0.003$) or recurrent angina (1307 [20.9 percent] vs. 1442 [22.9 percent]; relative risk, 0.91; 95 percent confidence interval, 0.85 to 0.98; $P = 0.01$) (Fig. 3). Slightly fewer patients in the clopidogrel group underwent coronary revascularization during the study (36.0 percent vs. 36.9 percent), but the difference was accounted for entirely by a difference in the rate of revascularization during the initial period of hospitalization (20.8 percent in the clopidogrel group vs. 22.7 percent in the placebo group, $P = 0.03$). Radiologic evidence of heart failure was found in fewer patients in the clopidogrel group (229 [3.7 percent], vs. 280 [4.4 percent] in the placebo group; relative risk, 0.82; 95 percent confidence interval, 0.69 to 0.98; $P = 0.03$).

Temporal Trends

The rate of the first primary outcome was lower in the clopidogrel group both within the first 30 days after randomization (relative risk, 0.79; 95 percent confidence interval, 0.67 to 0.92) and between 30 days and the end of the study (relative risk, 0.82; 95 percent confidence interval, 0.70 to 0.95) (Fig. 1 and 2). Further analysis indicated that the benefit of clopidogrel was apparent within a few hours after randomization, with the rate of death from cardiovascular causes, nonfatal myocardial infarction, stroke, or refractory or severe ischemia significantly lower in the clopidogrel group by 24 hours after randomization (1.4 percent in the clopidogrel group vs. 2.1 percent in

TABLE 1. BASE-LINE DEMOGRAPHIC CHARACTERISTICS, MEDICAL HISTORY, ELECTROCARDIOGRAPHIC CHANGES, AND DRUG THERAPY.*

CHARACTERISTIC	CLOPIDOGREL GROUP (N=6259)	PLACEBO GROUP (N=6303)
Age — yr	64.2±11.3	64.2±11.3
Female sex — no. (%)	2420 (38.7)	2416 (38.3)
Time from onset of pain to randomization — hr	14.2±7.2	14.1±7.1
Heart rate — beats/min	73.2±14.8	73.0±14.6
Systolic blood pressure — mm Hg	134.4±22.5	134.1±22.0
Diagnosis at study entry — no. (%)		
Unstable angina	4690 (74.9)	4724 (74.9)
Suspected myocardial infarction	1569 (25.1)	1579 (25.1)
Associated myocardial infarction — no. (%)†	1624 (25.9)	1659 (26.3)
Medical history — no. (%)		
Myocardial infarction	2029 (32.4)	2015 (32.0)
CABG or PTCA	1107 (17.7)	1139 (18.1)
Stroke	274 (4.4)	232 (3.7)
Heart failure	462 (7.4)	492 (7.8)
Hypertension	3750 (59.9)	3642 (57.8)
Diabetes	1405 (22.4)	1435 (22.8)
Current or former smoker	3790 (60.6)	3841 (60.9)
Electrocardiographic abnormality — no. (%)‡		
Any	5863 (93.7)	5921 (93.9)
ST segment		
Depression ≥1 mm	2642 (42.2)	2646 (42.0)
Elevation ≤1 mm	203 (3.2)	199 (3.2)
Transient elevation >2 mm	38 (0.6)	37 (0.6)
T-wave inversion		
Major (≥2 mm)	1589 (25.4)	1635 (25.9)
Other (<2 mm)	721 (11.5)	713 (11.3)
Other	670 (10.7)	690 (10.9)
Medications at time of randomization — no. (%)		
Aspirin	4168 (66.6)	4134 (65.6)
Heparin or LMW heparin	4522 (72.3)	4605 (73.1)
ACE inhibitor	2347 (37.5)	2309 (36.6)
Beta-blocker	3678 (58.8)	3690 (58.5)
Calcium-channel blocker	1784 (28.5)	1771 (28.1)
Lipid-lowering agent	1599 (25.6)	1586 (25.2)
Intravenous nitrate	2836 (45.3)	2906 (46.1)

*Plus-minus values are means ±SD. CABG denotes coronary-artery bypass grafting, PTCA percutaneous transluminal coronary angioplasty, LMW low molecular weight, and ACE angiotensin-converting enzyme.

†An associated myocardial infarction was defined as a myocardial infarction associated with the episode of pain that occurred before randomization.

‡Data on the particular type of abnormality were missing for one patient in the placebo group.

the placebo group; relative risk, 0.66; 95 percent confidence interval, 0.51 to 0.86).

Subgroup Analyses

The consistency of the results in a number of key subgroups is documented in Figure 4. The benefits were also consistent among subgroups receiving different doses of aspirin and among those receiving or not receiving lipid-lowering drugs, beta-blockers, heparin, or angiotensin-converting-enzyme inhibitors at

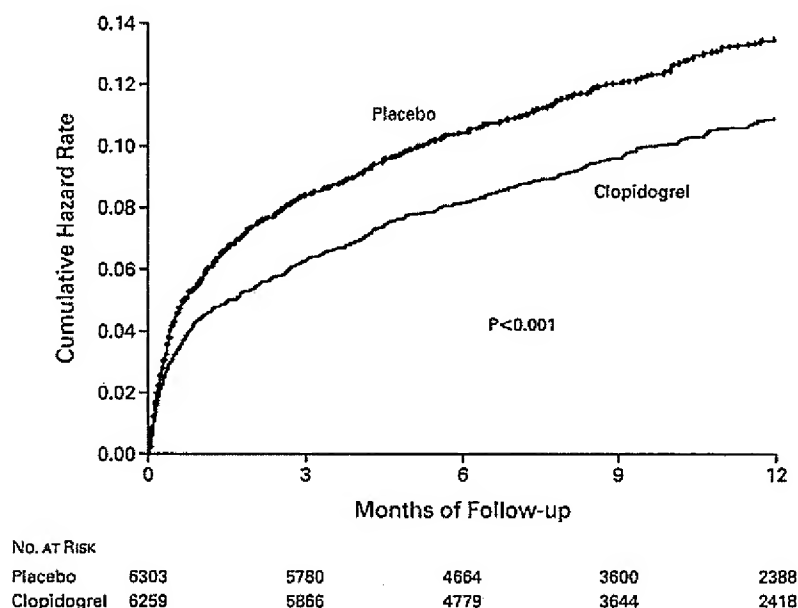


Figure 1. Cumulative Hazard Rates for the First Primary Outcome (Death from Cardiovascular Causes, Nonfatal Myocardial Infarction, or Stroke) during the 12 Months of the Study. The results demonstrate the sustained effect of clopidogrel.

the time of randomization. There was a tendency toward a greater benefit among patients who had previously undergone revascularization (relative risk of the first primary outcome, 0.56; 95 percent confidence interval, 0.43 to 0.72) than among those who had not (relative risk, 0.88; 95 percent confidence interval, 0.78 to 0.99; P for interaction = 0.002). However, these results should be interpreted cautiously, given the large numbers of subgroup analyses that were performed. Furthermore, consistent benefits were observed irrespective of whether patients underwent revascularization procedures after randomization.

Safety

Major bleeding was significantly more common in the clopidogrel group (3.7 percent in the clopidogrel group as compared with 2.7 percent in the placebo group; relative risk, 1.38; 95 percent confidence interval, 1.13 to 1.67; $P=0.001$) (Table 3). There were 135 patients with life-threatening bleeding episodes in the clopidogrel group (2.2 percent) as compared with 112 in the placebo group (1.8 percent; relative risk, 1.21; 95 percent confidence interval, 0.95 to 1.56). There was no excess rate of fatal bleeding, bleeding requiring surgical intervention, or hemorrhagic stroke. The excess major bleeding episodes were gastrointestinal hemorrhages and bleeding at the sites of

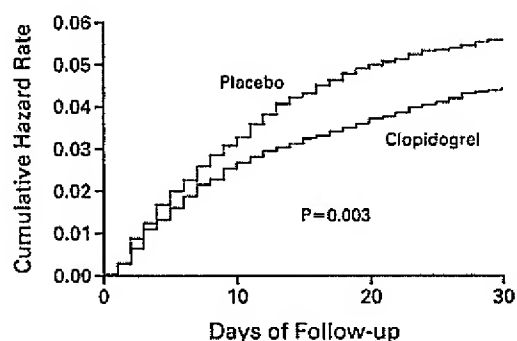


Figure 2. Cumulative Hazard Rates for the First Primary Outcome (Death from Cardiovascular Causes, Nonfatal Myocardial Infarction, or Stroke) during the First 30 Days after Randomization. The results demonstrate the early effect of clopidogrel.

TABLE 2. INCIDENCE OF THE MAIN STUDY OUTCOMES.*

OUTCOME	CLOPIDOGREL GROUP (N=6259)	PLACEBO GROUP (N=6303)	RELATIVE RISK (95% CI)	P VALUE
	no. (%)			
First primary outcome: nonfatal myocardial infarction, stroke, or death from cardiovascular causes	532 (9.3)	719 (11.4)	0.80 (0.72–0.90)	<0.001
Second primary outcome: first primary outcome or refractory ischemia	1035 (16.5)	1187 (18.8)	0.86 (0.79–0.94)	<0.001
Death from cardiovascular causes	318 (5.1)	345 (5.5)	0.93 (0.79–1.08)	
Myocardial infarction†	324 (5.2)	419 (6.7)	0.77 (0.67–0.89)	
Q-wave	116 (1.9)	193 (3.1)	0.60 (0.48–0.76)	
Non-Q-wave	216 (3.5)	242 (3.8)	0.89 (0.74–1.07)	
Stroke	75 (1.2)	87 (1.4)	0.86 (0.63–1.18)	
Refractory ischemia‡	544 (8.7)	587 (9.3)	0.93 (0.82–1.04)	
During initial hospitalization	85 (1.4)	126 (2.0)	0.68 (0.52–0.90)	
After discharge	459 (7.6)	461 (7.6)	0.99 (0.87–1.13)	
Death from noncardiovascular causes	41 (0.7)	45 (0.7)	0.91 (0.60–1.39)	

*The number of patients who died from cardiovascular causes or had a nonfatal myocardial infarction was 539 (8.6 percent) in the clopidogrel group and 660 (10.5 percent) in the placebo group ($P<0.001$; relative risk, 0.81; 95 percent confidence interval, 0.72 to 0.91). The corresponding numbers at 30 days were 241 (3.9 percent) and 305 (4.8 percent) (relative risk, 0.79; 95 percent confidence interval, 0.67 to 0.94; $P=0.007$). CI denotes confidence interval.

†Some patients had both a Q-wave and a non-Q-wave myocardial infarction.

‡Only the first ischemic event was counted for each patient.

arterial punctures. The number of patients who required the transfusion of 2 or more units of blood was higher in the clopidogrel group (177 [2.8 percent]) than in the placebo group (137 [2.2 percent], $P=0.02$). The rate of major bleeding episodes was higher early (within 30 days after randomization: 2.0 percent vs. 1.5 percent; relative risk, 1.31; 95 percent confidence interval, 1.01 to 1.70) and also late (more than 30 days after randomization: 1.7 percent vs. 1.1 percent; relative risk, 1.48; 95 percent confidence interval, 1.10 to 1.99). Overall, there was no significant excess of major bleeding episodes after coronary-artery bypass grafting (CABG) (1.3 percent vs. 1.1 percent; relative risk, 1.26; 95 percent confidence interval, 0.93 to 1.71). However, in most patients scheduled for CABG surgery, the study medication was discontinued before the procedure (median time before the procedure, five days). In the 910 patients in whom the study medication was discontinued more than five days before the procedure (five days being the duration of the effect of clopidogrel), there was no apparent excess of major bleeding within seven days after surgery (4.4 percent of the patients in the clopidogrel group vs. 5.3 percent of those in the placebo group). In the 912 patients who stopped taking the medications within five days before CABG surgery, the rate of major bleeding was 9.6 percent in the clopidogrel group and 6.3 percent in the placebo group (relative risk, 1.53;

$P=0.06$). Overall, the risk of minor bleeding was significantly higher in the clopidogrel group than in the placebo group (322 [5.1 percent] vs. 153 [2.4 percent]; $P<0.001$). The numbers of patients with thrombocytopenia (28 in the placebo group and 26 in the clopidogrel group) or neutropenia (5 and 8, respectively) were similar.

Adherence to Study Medication and Aspirin

A total of 46.2 percent of the patients in the clopidogrel group discontinued the study medication temporarily (for more than five days), as compared with 45.4 percent in the placebo group. The most common reason for the temporary discontinuation of the study medication was the need for revascularization or another surgical procedure; 84 percent of the patients with such a need discontinued the medication before the procedure. A total of 21.1 percent of the patients in the clopidogrel group discontinued the study medication permanently, as compared with 18.8 percent in the placebo group. A total of 99 percent of the patients in both groups were taking aspirin while they were in the hospital, 96 percent were taking it at three months, and 94 percent at the final visit. The use of all other medications (other than thrombolytic therapy and glycoprotein IIb/IIIa receptor inhibitors) was similar in the clopidogrel group and the placebo group.

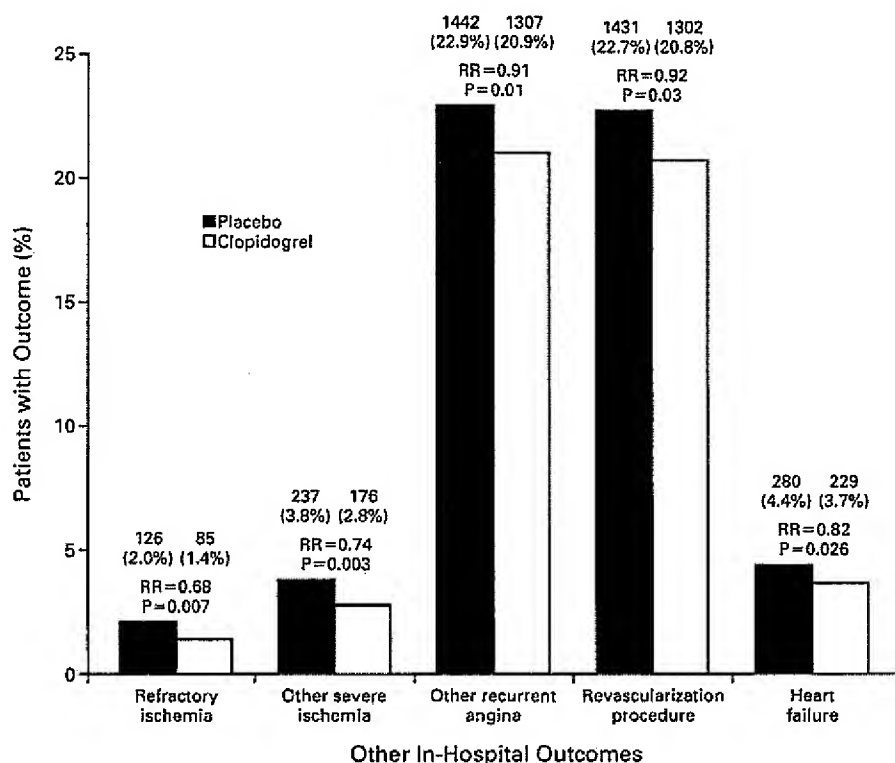


Figure 3. Proportions of Patients Who Had Events Other Than Those Included in the First Primary Outcome while They Were in the Hospital.

The numbers and percentages of patients in each group with the specified outcome are given above the bars. RR denotes relative risk.

Thrombolytic Therapy and Glycoprotein IIb/IIIa Receptor Inhibitors

A total of 71 patients in the clopidogrel group (1.1 percent) and 126 patients in the placebo group (2.0 percent) received thrombolytic therapy (relative risk, 0.57; 95 percent confidence interval, 0.43 to 0.76; $P < 0.001$); 369 patients in the clopidogrel group (5.9 percent) and 454 in the placebo group (7.2 percent) received a glycoprotein IIb/IIIa receptor inhibitor (relative risk, 0.82; 95 percent confidence interval, 0.72 to 0.93; $P = 0.003$).

DISCUSSION

Our study demonstrates the benefit of adding clopidogrel to the regimen of treatment for patients with acute coronary syndromes without ST-segment elevation who are receiving aspirin and other medications. Treatment with clopidogrel reduced the risk of myocardial infarction and recurrent ischemia, with a trend toward lower rates of stroke and death from cardiovascular causes. Fewer patients in the clopidogrel group received a thrombolytic agent or an intravenous gly-

coprotein IIb/IIIa receptor inhibitor. The benefits we observed were in addition to those of aspirin, which was recommended for all patients, indicating that blocking the adenosine diphosphate-receptor pathway with clopidogrel leads to further benefit.

Our study primarily included centers in which there was no routine policy of early use of invasive procedures, since such a policy would have led to a high rate of immediate discontinuation of the study medication and the use of an open-label thienopyridine derivative. Once a patient had been randomly assigned to a treatment group, there were no restrictions on the use of any therapy or intervention. In particular, if the clinician believed that angiography and revascularization were needed or that a thienopyridine derivative was indicated, the study medication could be stopped or open-label clopidogrel or ticlopidine could be used. In fact, 5491 patients (43.7 percent) underwent angiography, 2072 patients (16.5 percent) underwent CABG, and 2658 patients (21.2 percent) underwent PTCA. In 85.8 percent of the patients who underwent PTCA and 84.9 percent of those who underwent

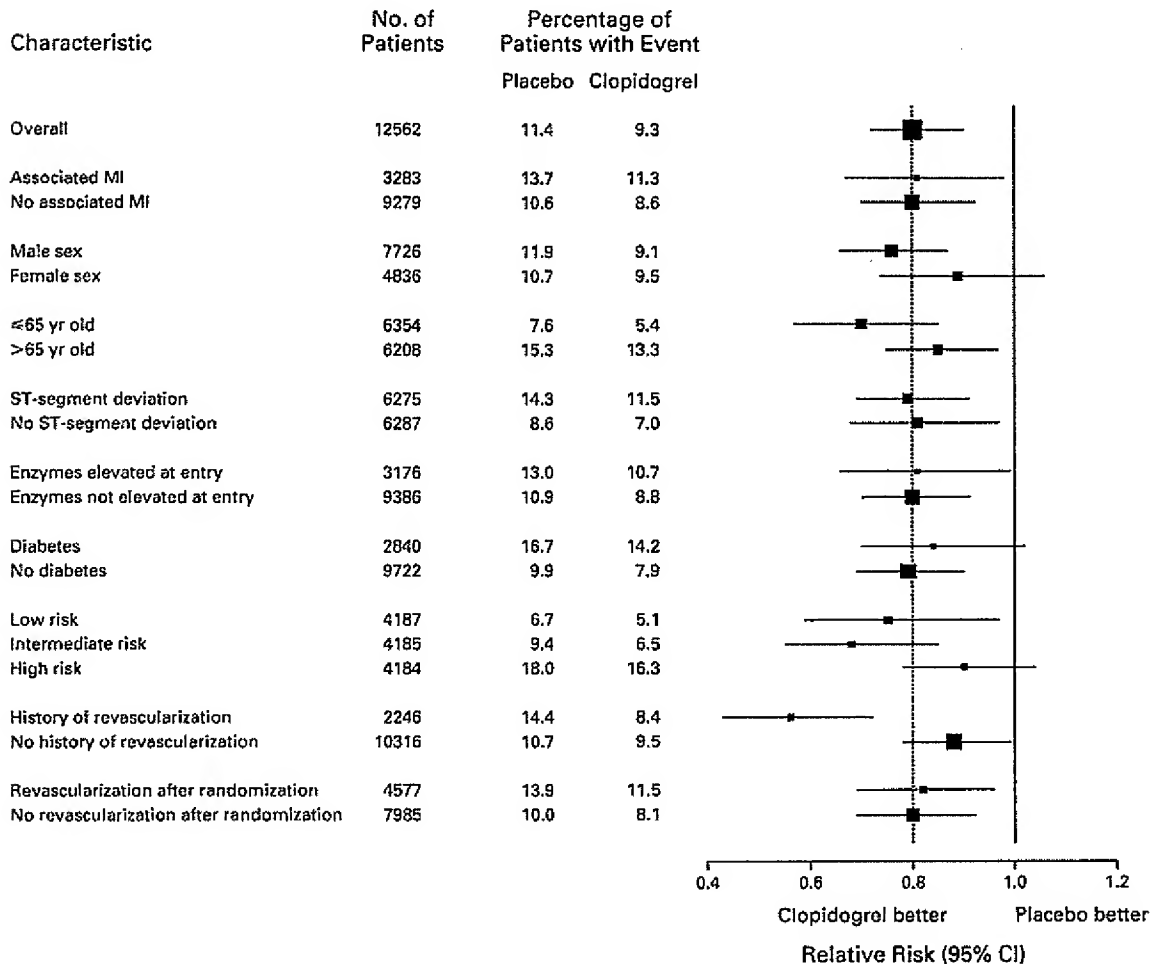


Figure 4. The Rates and Relative Risks of the First Primary Outcome (Death from Cardiovascular Causes, Nonfatal Myocardial Infarction, or Stroke) in Various Subgroups.

The data show the consistency of the benefit of clopidogrel. The dotted line represents the average treatment effect. The size of each box is proportional to the number of patients in the individual analysis. An associated myocardial infarction (MI) was defined as a myocardial infarction associated with the episode of pain that occurred before randomization. Because of missing data, six patients could not be classified in a risk category. CI denotes confidence interval.

CABG, the use of the study medication was temporarily interrupted for more than five days, and the vast majority of the patients who underwent PTCA received a thienopyridine-type antiplatelet agent for about two to four weeks. In the patients who underwent CABG, the study medication was restarted after a median of 11 days. Although these interruptions of therapy with the study medication would tend to result in an underestimate of the difference between the clopidogrel group and the placebo group, they also permit us to make useful estimates of the benefits and risks of clopidogrel when it is used routinely and over the long term, as compared with a strategy of more

selective and short-term use among those undergoing implantation of a coronary stent.

Clopidogrel prevented a range of ischemic coronary events — among them, myocardial infarction and severe and refractory ischemia. Clopidogrel was associated with a trend toward fewer ischemic strokes, and there was no increase in the rate of hemorrhagic stroke that would offset these benefits. There was a significant reduction in the incidence of heart failure with clopidogrel that was of about the same magnitude as the reduction in the incidence of ischemic events, suggesting that the reduction of ischemia can prevent heart failure. The benefits of clopidogrel were observed in

TABLE 3. BLEEDING COMPLICATIONS.*

VARIABLE	CLOPIDOGREL GROUP (N=6259)	PLACEBO GROUP (N=6303)	RELATIVE RISK (95% CI)	P VALUE
	no. (%)			
Major bleeding	231 (3.7)	169 (2.7)	1.38 (1.13–1.67)	0.001
Necessitating transfusion of ≥ 2 units of blood	177 (2.8)	137 (2.2)	1.30 (1.04–1.62)	0.02
Life-threatening	135 (2.2)	112 (1.8)	1.21 (0.95–1.56)	0.13
Fatal	11 (0.2)	15 (0.2)		
Causing 5 g/dl drop in hemoglobin level	58 (0.9)	57 (0.9)		
Requiring surgical intervention	45 (0.7)	43 (0.7)		
Causing hemorrhagic stroke	7 (0.1)	5 (0.1)		
Requiring inotropic agents	34 (0.5)	34 (0.5)		
Necessitating transfusion of ≥ 4 units of blood	74 (1.2)	60 (1.0)		
Non-life-threatening	96 (1.5)	57 (0.9)	1.70 (1.22–2.35)	0.002
Site of major bleeding				
Gastrointestinal	83 (1.3)	47 (0.7)		
Retroperitoneal	8 (0.1)	5 (0.1)		
Urinary (hematuria)	4 (0.1)	5 (0.1)		
Arterial puncture site	36 (0.6)	22 (0.3)		
Surgical site	56 (0.9)	53 (0.8)		
Minor bleeding	322 (5.1)	153 (2.4)	2.12 (1.75–2.56)	<0.001
Total with bleeding complications	533 (8.5)	317 (5.0)	1.69 (1.48–1.94)	<0.001

*The number of patients with bleeding that met the criteria for major bleeding established by the Thrombolysis in Myocardial Infarction trial¹¹ was 68 in the clopidogrel group and 73 in the placebo group (relative risk, 0.94; 95 percent confidence interval, 0.68 to 1.30; $P=0.70$). The number with bleeding that met the criteria for life-threatening or severe bleeding established by the Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries trial¹² was 78 in the clopidogrel group and 70 in the placebo group (relative risk, 1.12; 95 percent confidence interval, 0.81 to 1.55; $P=0.48$). Some patients had more than one bleeding episode. CI denotes confidence interval.

a range of patients, including both patients who were undergoing revascularization procedures and those who were not. The benefits were also observed in those at low, medium, and high risk of cardiovascular events and those who were receiving various proven therapies such as aspirin, lipid-lowering drugs, angiotensin-converting-enzyme inhibitors, and beta-blockers.^{13–16} The benefits of clopidogrel were apparent as early as the first 24 hours after randomization, indicating that the oral loading dose was rapidly effective. Thereafter, the differences between the two groups were maintained until the end of the study.

Clopidogrel increased the risk of minor and major bleeding episodes. For every 1000 patients treated with clopidogrel, 6 will require a transfusion. However, there was no excess in bleeding that caused strokes, required surgical intervention or inotropic agents, or caused permanent disability. Furthermore, the excess risk of bleeding we observed is similar to that observed with aspirin alone, as compared with a control, in previous studies and lower than that observed in most trials of the short-term intravenous use or the prolonged oral use of glycoprotein IIb/IIIa receptor inhibitors.^{4,16,17} The risk of bleeding may have been partly mitigated by the temporary discontinuation of the study medication before surgery. Treatment with clo-

pidogrel was not associated with an excess rate of any other type of adverse event that necessitated the discontinuation of the study drug; this finding indicates that the combination of clopidogrel and aspirin is as well tolerated as aspirin alone.

In summary, clopidogrel significantly reduces the risk of the composite outcome of death from cardiovascular causes, nonfatal myocardial infarction, or stroke, as well as a range of related ischemic events. The use of the drug, in addition to aspirin, is associated with an increased risk of bleeding.

Supported by Sanofi-Synthelabo and Bristol-Myers Squibb. Dr. Yusuf is the recipient of a Senior Scientist award from the Canadian Institutes of Health Research and holds an endowed chair from the Heart and Stroke Foundation of Ontario. Dr. Mehta is a research fellow of the Heart and Stroke Foundation of Canada.

Drs. Yusuf, Mehta, Tognoni, and Fox have received honorariums for educational activities or have served as consultants to Bristol-Myers Squibb and Sanofi-Synthelabo.

We are indebted to Judy Lindeman for secretarial assistance.

APPENDIX

The following persons participated in the CURE trial: Steering Committee: S. Yusuf (chair and principal investigator), K.A.A. Fox (cochair), G. Tognoni (cochair), S.R. Mehta (project officer), S. Chrolavicius (study coordinator), S. Anand, A. Avezum, N. Awan, M. Bertrand, A. Budaj, L.

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CORRECTION

**Effects of Clopidogrel in Addition to Aspirin in
Patients with Acute Coronary Syndromes without
ST-Segment Elevation**

Effects of Clopidogrel in Addition to Aspirin in Patients with Acute
Coronary Syndromes without ST-Segment Elevation . On page 502,
20 lines from the bottom of the left-hand column, "B. Pontillo" should
have been listed as "D. Pontillo."

CORRECTION

Effects of Clopidogrel in Addition to Aspirin in Patients with Acute Coronary Syndromes without ST-Segment Elevation

Effects of Clopidogrel in Addition to Aspirin in Patients with Acute Coronary Syndromes without ST-Segment Elevation . On page 494, at the end of the Results section of the abstract, the percentages of patients with episodes of life-threatening bleeding should have been "2.2 percent vs. 1.8 percent," not "2.1 percent vs. 1.8 percent," as printed. In the Manuscript Writing Committee listed at the bottom of the right-hand column, "Keith K. Fox" should have read "Keith A.A. Fox." On page 498, on line 13 of the left-hand column, the rates of bleeding after coronary-artery bypass grafting should have read, "8.3 percent vs. 6.6 percent," not "7.3 percent vs. 7.1 percent," as printed.

Exhibit 21

The *in vivo* pharmacological profile of CS-747, a novel antiplatelet agent with platelet ADP receptor antagonist properties

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1 CS-747 is a novel antiplatelet agent that generates an active metabolite, R-99224, *in vivo*. CS-747 itself was totally inactive *in vitro*. This study examined *in vivo* pharmacological profiles of CS-747 after single oral administration to rats.

2 Orally administered CS-747 (0.3–10 mg kg⁻¹) partially but significantly decreased [³H]-2-methylthio-ADP binding to rat platelets. CS-747 (3 mg kg⁻¹, p.o.) treatment neutralized ADP-induced decreases of cyclic AMP concentrations induced by prostaglandin E₁, suggesting that metabolites of CS-747 interfere with G_i-linked P2T receptor.

3 CS-747 (0.3 and 3 mg kg⁻¹, p.o.) markedly inhibited *ex vivo* washed platelet aggregation in response to ADP but not to thrombin. CS-747 also exhibited a marked inhibition of ADP-induced *ex vivo* platelet aggregation in PRP with a rapid onset (<0.5 h) and long duration (>3 days) of action (ED₅₀ at 4 h = 1.2 mg kg⁻¹).

4 R-99224 (IC₅₀ = 45 µM) inhibited *in vitro* PRP aggregation in a concentration-related manner.

5 CS-747 prevented thrombus formation in a dose-related manner with an ED₅₀ value of 0.68 mg kg⁻¹. CS-747 was more potent than clopidogrel (6.2 mg kg⁻¹) and ticlopidine (>300 mg kg⁻¹).

6 CS-747, clopidogrel, and ticlopidine prolonged the bleeding time. The order of potency of these agents in this activity was the same as that in antiaggregatory and antithrombotic activities.

7 These findings indicate that CS-747 is an orally active and a potent antiplatelet and antithrombotic agent with a rapid onset and long duration of action, and warrants clinical evaluations of the agent.

British Journal of Pharmacology (2000) 129, 1439–1446

Keywords: Platelet aggregation; thrombosis; bleeding time; CS-747; platelet ADP receptors; active metabolite; R-99224

Abbreviations: ADP, adenosine 5'-diphosphate; BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; IBMX, 3-isobutyl-1-methylxanthine; 2-MeS-ADP, 2-methylthio-adenosine 5'-diphosphate; PGE₁, prostaglandin E₁; PPP, platelet-poor plasma; PRP, platelet-rich plasma

Introduction

The platelet activation and subsequent platelet aggregation play an essential role in the pathogenesis of cardiovascular, cerebrovascular, and peripheral vascular diseases (Antiplatelet Trialists' Collaboration, 1988; Schrör, 1995). Upon vascular injury, ADP, a potent platelet activator, is released into the bloodstream from damaged cells and activated platelets, which in turn acts on other platelets (Gachet *et al.*, 1996). ADP induces a number of responses in platelets, including shape change from disc to sphere, aggregation, and secretion of granule contents (Gachet & Cazenave, 1991). These responses are considered to be mediated by ADP's interaction with specific binding sites on the platelet membrane that have been tentatively designated as P2T receptors (Hourani & Hall, 1994; Gachet *et al.*, 1996). The P2T receptors are probably composed of three distinct receptors, i.e., the P2X₁, ligand gated ion channel receptors and two distinct G-protein coupled ADP receptors (a G_i-linked P2Y₁ receptor and a G_i-linked P2T receptor distinct from P2Y₁) (for review see Kunapuli, 1998a, b).

The importance of the G_i-linked P2T receptor in platelet aggregation has been demonstrated in recent studies using ARL 66096, ticlopidine and clopidogrel (Mills *et al.*, 1992;

Fagura *et al.*, 1998; Daniel *et al.*, 1998). Thienopyridine derivatives such as ticlopidine and clopidogrel are orally active inhibitors of ADP-induced platelet aggregation with a slow onset and long duration of action (McTavish *et al.*, 1990; Coukell & Markham, 1997). Previous studies have demonstrated that these agents inhibit the binding of radiolabelled 2-methylthio-ADP (2-MeS-ADP), a stable analogue of ADP, to human and animal platelets *ex vivo*. It has been speculated that the mechanism of those actions involves the inhibition of the G_i-linked P2T receptor (Mills *et al.*, 1992; Savi *et al.*, 1994a; Gachet *et al.*, 1995), but the putative active metabolites remain to be elucidated (Saltiel & Ward, 1987; Savi *et al.*, 1994b; Coukell & Markham, 1997). The clinical efficacy of these agents was demonstrated in human clinical trials, e.g. CATS (Gent *et al.*, 1989), TASS (Hass *et al.*, 1989) and CAPRIE (CAPRIE Steering Committee, 1996). However, the therapeutic doses of ticlopidine are accompanied by serious side effects such as neutropenia and abnormal liver function. Clopidogrel has been reported to be as safe as aspirin, but its therapeutic benefit compared to aspirin is marginal (CAPRIE Steering Committee, 1996).

CS-747 (Figure 1) is a novel antiplatelet agent which generates an active metabolite, R-99224 (Figure 1), *in vivo*. In the present studies, we investigated the *in vivo* pharmacological profile of CS-747 in rats. In addition, we compared the

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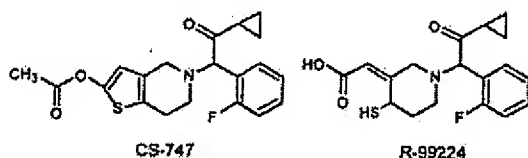


Figure 1 Chemical structures of CS-747 and its active metabolite, R-99224.

antiplatelet and antithrombotic effects of single oral administrations of CS-747 to those of clopidogrel and ticlopidine. The pharmacological profile of CS-747 revealed in the present study shows its potential as an antiplatelet agent.

Methods

Animals

The experimental procedures employed in this study were in accordance with the guidelines of the Institutional Animal Care and Use Committee at Sankyo Research Laboratories (Tokyo, Japan). We used male Sprague-Dawley rats purchased from Japan SLC (Shizuoka, Japan). The animals were allowed free access to standard rat chow and water.

Preparation of platelet-rich plasma and washed platelets

Rats were anaesthetized with pentobarbital sodium (40 mg kg⁻¹, i.p.). Blood was drawn from the abdominal aorta into a plastic syringe containing 3.8% (w/v) trisodium citrate (1:9 volumes of blood) as an anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation at 230 × g for 15 min at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at 2000 × g for 10 min. Platelet counts in PRP were adjusted to 5 × 10⁸ ml⁻¹ by adding PPP.

Washed platelets were prepared as described previously (Sugidachi *et al.*, 1998) with slight modifications. After prostaglandin E₁ (PGE₁) at 100 nM was added to the PRP, the mixture was centrifuged at 1300 × g for 6 min, and the resulting platelet pellet was resuspended in a washing buffer containing (in mM): NaCl 140, KCl 2.7, NaHCO₃ 12, NaH₂PO₄ 0.4, MgCl₂ 0.8, glucose 5, HEPES 10, and 3.5 mg ml⁻¹ fatty acid-free bovine serum albumin, pH 6.7. Finally this platelet suspension was further washed and resuspended in the suspension buffer (same composition as the washing buffer, pH 7.4). In studies on washed platelets, the platelet suspension was supplemented with 0.068 mg ml⁻¹ human fibrinogen and 1 mM Ca²⁺.

[³H]-2-MeS-ADP binding

The washed platelet suspension (2 × 10⁸ platelets ml⁻¹) was incubated with 10 nM [³H]-2-MeS-ADP at room temperature. After 30 min, the reaction mixture was layered onto a 20% sucrose solution in suspension buffer and the bound ligand was separated by centrifugation at 10,000 × g for 3 min at room temperature. After careful aspiration of the supernatant, the platelet pellet was dissolved in NCS-II (Amersham, Buckinghamshire, U.K.) and its radioactivity was measured by scintillation counting. Specific binding was defined as the difference between the total binding and nonspecific binding determined by addition of unlabelled 2-MeS-ADP at 100 μM.

Measurement of cyclic AMP concentration

To indirectly measure adenylyl cyclase activity, cyclic AMP levels were determined according to the method of Defrey *et al.* (1991). PGE₁ was used to elevate cyclic AMP levels. A mixture of 1.5 ml buffer (in mM: Tris 15, NaCl 120, KCl 4, MgSO₄ 1.6, NaH₂PO₄·2H₂O 2, glucose 10, 0.2% BSA, IBMX 1.5; pH 7.4) and 3 ml PRP (5 × 10⁸ platelets ml⁻¹) was incubated at 37°C for 1 min, and then PGE₁ (10 μM) was added. ADP (10 μM) or saline was added to the reaction mixture at 3 min after PGE₁ stimulation. Aliquots in a volume of 0.5 ml were taken from the reaction mixture before and 1, 3, 4, and 6 min after PGE₁ stimulation. These samples were supplemented with 50 μl of 6N HCl and 50 μM EDTA solution and boiled for 5 min. After rapid cooling on ice, the samples were centrifuged at 10,000 × g for 5 min at 4°C. After adding CaCO₃ (60 mg), the supernatants (300 μl) were incubated at room temperature for 15 min and then centrifuged again at 10,000 × g for 5 min at 4°C. The final supernatants were assayed for cyclic AMP levels using a commercially available EIA kit (Amersham, Buckinghamshire, U.K.).

Measurement of ex vivo platelet aggregation and shape change

All aggregation studies were performed in Mechani aggregometers (model PAM-6C and PAM-8C, Tokyo, Japan). The washed platelets (3 × 10⁸ platelets ml⁻¹) or PRP (5 × 10⁸ platelets ml⁻¹) in a volume of 240 μl were incubated at 37°C for 1.5 min in the aggregometer with continuous stirring at 1000 r.p.m. and then stimulated with 10 μl of ADP, collagen, or thrombin. Changes in light transmission were recorded for 7 min (ADP) and for 10 min (collagen and thrombin) after stimulation with these agents. The extent of aggregation was expressed as a percentage of the maximum light transmittance, obtained with the suspension buffer (washed platelet aggregation) or PPP (PRP aggregation). Platelet shape change was determined using an aggregometer, PAM-6C according to the method by Michal & Motamed (1976), and was estimated quantitatively by measuring the maximum height above baseline level.

Arterio-venous shunt thrombosis model

The ability of test agents to prevent thrombus formation was assessed using an arterio-venous shunt model originally described by Umetsu & Sanai (1978) with slight modifications. After anaesthesia with pentobarbital sodium (40 mg kg⁻¹, i.p.), the jugular vein and contralateral carotid artery of rats were exposed and they were cannulated with a polyethylene cannula which contains a silk thread in its lumen and is filled with heparin solution (30 unit kg⁻¹). Blood circulation was started through the cannula allowing thrombus formation to occur on the silk thread. After a 30 min circulation, the cannula tube was removed and the silk thread was weighed. The weight of thrombus formed on the thread was calculated by deducting the wet weight of an equivalent length of the standard thread. Drugs or vehicle were administered 4 h before starting blood flow.

Bleeding time

The tail transection bleeding time was determined by the method of Dejana *et al.* (1979). The test drugs were orally administered 4 h before the tail transection. Under anaesthesia with pentobarbital (40 mg kg⁻¹, i.p.), the rat tail was

transected at 4 mm from the tip by a scalpel, and the tail was immediately immersed into warmed (37°C) saline until blood flow stopped. Bleeding time was assessed as the time from the tail transection to the termination of blood flow. Bleeding times beyond 1800 s were regarded as 1800 s for the purpose of statistical analysis. The BT_2 was defined as the dose that doubled the control bleeding time.

Drugs and administration

CS-747 (2-acetoxy-5-(α -cyclopropylcarbonyl-2-fluorobenzyl)-4,5,6,7-tetrahydrothieno [3,2-c]pyridine), R-99224 ((2Z)-[1-(2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl)-4-mercapto-3-piperidinylidene] acetic acid, trifluoroacetate), and clopidogrel hydrogensulphate (SR25990C) were synthesized by Ube Industries (Yamaguchi, Japan). Ticlopidine hydrochloride, ADP (sodium salt), human fibrinogen, fatty-acid-free bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine (IBMX), ethylenediaminetetraacetic acid (EDTA), N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES), apyrase and gum arabic were purchased from Sigma (St. Louis, MO, U.S.A.). Collagen was from Nycomed (Munich, Germany), heparin (sodium salt) was from Fuso (Osaka, Japan). PGE₁ was from Funakoshi (Tokyo, Japan), and bovine thrombin was from Mochida (Tokyo, Japan). [³H]-2-MeS-ADP (ammonium salt, specific activity 85 Ci mmol⁻¹) was obtained from Amersham (Buckinghamshire, U.K.), and 2-MeS-ADP (trisodium salt) was obtained from Research Biochemicals International (Natick, MA, U.S.A.).

CS-747, clopidogrel, and ticlopidine were suspended in 5% gum arabic solution at appropriate concentrations and orally administered to rats in a volume of 1 ml kg⁻¹ body weight. All experiments included the vehicle-treated control groups. In *in vitro* study, R-99224 and CS-747 were dissolved in DMSO, and added to the PRP; the final concentration of DMSO was 0.4%.

Statistics

Results were expressed as mean \pm s.e.mean. Approximate estimate of the ED₅₀ and BT_2 values were calculated from linear-regression analysis. Dunnett's test for multiple comparison was used to determine significance of differences between mean values within groups. A *P* value of less than 0.05 was considered statistically significant.

Results

[³H]-2-MeS-ADP binding

Preliminary studies showed that [³H]-2-MeS-ADP binding to rat platelets was time-related and saturable, with an apparent equilibrium dissociation constant (*K*_d) of 2.37 ± 0.27 nM and a total number of receptor sites (*B*_{max}) of 180.2 ± 13.8 fmol 10⁸ platelets⁻¹ (*n*=4). *In vitro* treatment with CS-747 (100 and 300 μ M) had no effects on [³H]-2-MeS-ADP binding to the washed platelets of rats (data not shown).

Ex vivo effects of CS-747 (0.3–10 mg kg⁻¹, p.o.) and clopidogrel (3–100 mg kg⁻¹, p.o.) on [³H]-2-MeS-ADP (10 nM) binding to rat platelets were examined 4 h after dosing. After 30 min incubation, the specific [³H]-2-MeS-ADP binding to platelets from vehicle-treated control rats was 134.4 ± 15.2 fmol 10⁸ platelets⁻¹ (*n*=6). As shown in Figure 2, [³H]-2-MeS-ADP binding was significantly (*P*<0.01) decreased in platelets from rats given CS-747 (1–10 mg kg⁻¹, p.o.). But the inhibition remained partial (approximately 43%)

even at a dose of CS-747 as high as 10 mg kg⁻¹. Orally administered clopidogrel also showed similar partial inhibition of [³H]-2-MeS-ADP binding to rat platelets, but clopidogrel was about ten times less potent than CS-747 (Figure 2).

Cyclic AMP levels in platelets

Since ADP inhibits adenylyl cyclase via activation of G_i protein (Defrey *et al.*, 1991; Ohlmann *et al.*, 1995), we examined the *ex vivo* effects of CS-747 on ADP-mediated suppression of PGE₁-induced cyclic AMP elevation. An addition of PGE₁ (10 μ M) produced a progressive increase of intraplatelet cyclic AMP levels, indicating the activation of adenylyl cyclase. The elevated cyclic AMP levels were suppressed by ADP (10 μ M) added 3 min after PGE₁ stimulation. As shown in Figure 3, the inhibitory effect of ADP (10 μ M) on elevated cyclic AMP levels was inhibited substantially in platelets from CS-747 (3 mg kg⁻¹, p.o.)-treated rats. There was no difference in the basal cyclic AMP levels between CS-747-treated (0.39 ± 0.15 nmol 10⁸ platelets⁻¹, *n*=5) and vehicle-treated platelets (0.37 ± 0.06 nmol 10⁸ platelets⁻¹, *n*=5).

Ex vivo and in vitro platelet aggregation

Washed platelets To eliminate possible fibrin production induced by thrombin, experiments to test agonist selectivity

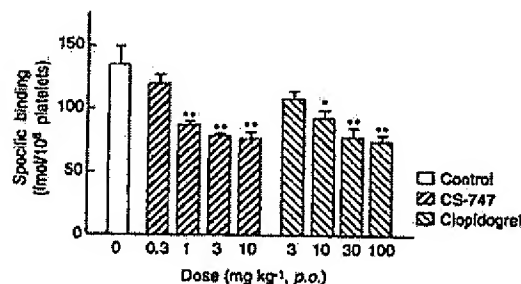


Figure 2 *Ex vivo* effects of CS-747 on specific [³H]-2-MeS-ADP binding to rat washed platelets. CS-747 (0.3–10 mg kg⁻¹) and clopidogrel (3–100 mg kg⁻¹) were orally administered to rats 4 h before blood collection. Results are expressed as the mean \pm s.e.mean (*n*=6). **P*<0.05, ***P*<0.01 vs control.

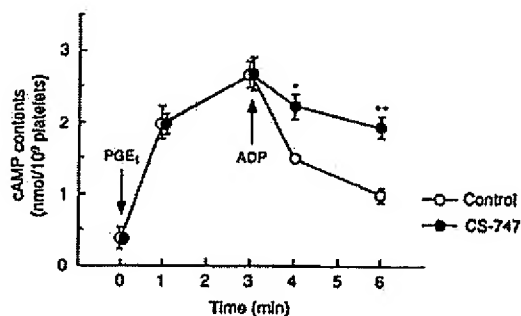


Figure 3 *Ex vivo* effects of CS-747 on ADP (10 μ M)-induced cyclic AMP decrease in PGE₁ (10 μ M)-stimulated rat platelets. CS-747 (3 mg kg⁻¹) was orally administered to rats 4 h before blood collection. Results are expressed as the mean \pm s.e.mean (*n*=5). **P*<0.05, ***P*<0.01 vs control.

was performed using washed platelets. A single oral administration of CS-747 (0.3 and 3 mg kg⁻¹) produced a dose-related inhibition of ADP (0.3–30 µM)-induced *ex vivo* aggregation in washed platelets (Figure 4A). Collagen (0.3–3 µg ml⁻¹)-induced aggregation was also inhibited by CS-747 administration in a dose-related manner (Figure 4B). In contrast, CS-747 moderately inhibited platelet aggregation induced by a low concentration of thrombin (0.06 unit ml⁻¹), but not that by high concentrations of thrombin (0.1 and 0.3 unit ml⁻¹) (Figure 4C). Apyrase (0.3 and 3 IU ml⁻¹) also inhibited platelet aggregation induced by ADP (30 µM) and collagen (3 µg ml⁻¹), but not by thrombin (0.3 unit ml⁻¹) (data not shown).

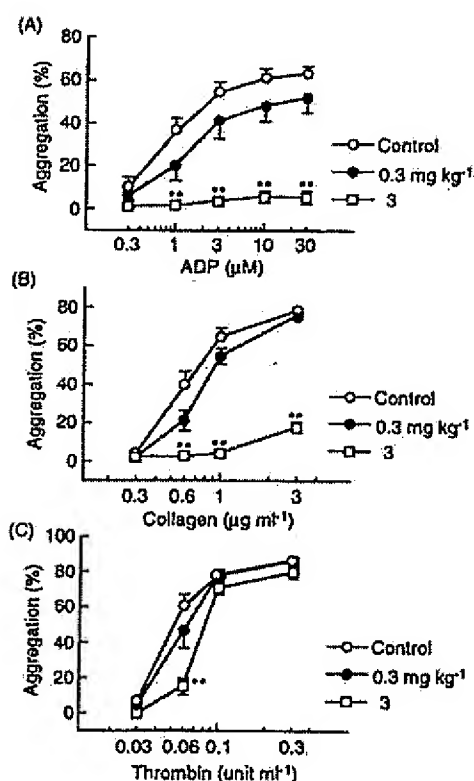


Figure 4 *Ex vivo* effects of single administration of CS-747 on washed platelet aggregation induced by ADP (A), collagen (B), and thrombin (C) in rats. CS-747 was orally administered once to rats at doses of 0.3 and 3 mg kg⁻¹. The aggregation was measured 4 h after the dosing. Results are presented as the mean \pm s.e. mean ($n=6$). ** $P<0.01$ vs control (vehicle-treated group).

Platelet-rich plasma (PRP) PRP was prepared from rats administered with a single dose of CS-747 (0.3–3 mg kg⁻¹, p.o.). Figure 5 shows the *ex vivo* effects of CS-747 on a concentration-aggregation curve for ADP determined at 4 h post-dose. We also investigated the antiaggregatory effects of clopidogrel and ticlopidine, two existing platelet ADP receptor inhibitors. A single oral administration of clopidogrel (3–300 mg kg⁻¹) caused a dose-related inhibition of ADP-induced aggregation, but the inhibitory effects of ticlopidine (30–300 mg kg⁻¹) were minimal. Table 1 summarizes ED₅₀ values for CS-747 (1.2 mg kg⁻¹), clopidogrel (16 mg kg⁻¹) and ticlopidine (>300 mg kg⁻¹) against ADP (3 µM)-induced platelet aggregation determined at 4 h post-dose. Typical tracing of platelet aggregation induced by ADP (3 µM) in PRP from rats treated with CS-747 is shown in Figure 6. As can be seen, CS-747 (1 and 3 mg kg⁻¹, p.o.) suppressed the maximum extent of platelet aggregation in a dose-related manner. Similar effects on platelet aggregation were obtained with clopidogrel and ticlopidine (data not shown).

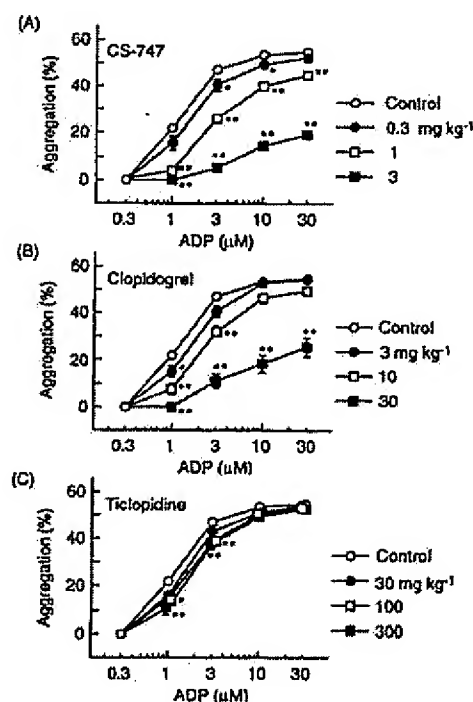


Figure 5 *Ex vivo* effects of single administration of CS-747 (A), clopidogrel (B), and ticlopidine (C) on ADP-induced platelet aggregation in rats. The aggregation in PRP was measured 4 h after the oral dosing. Results are presented as the mean \pm s.e. mean ($n=6$). * $P<0.05$, ** $P<0.01$ vs control (vehicle-treated group).

Table 1 Comparison of antiplatelet, antithrombotic, and antihemostatic effects of CS-747, clopidogrel and ticlopidine

Agents	AV-shunt (ED _{50AV})	Aggregation (ED _{50AGG})	Bleeding (BT ₂)	Ratio (ED _{50AV} /BT ₂)	Ratio (ED _{50AGG} /BT ₂)
CS-747	0.68	1.2	0.50	1.4	2.4
Clopidogrel	6.2	16	4.6	1.3	3.5
Ticlopidine	>300	>300	130	>2.3	>2.3

Agents were orally administered to rats 4 h before tests. In platelet aggregation (AGG) and the arterio-venous shunt thrombosis model (AV), the ED₅₀ values (mg kg⁻¹) are doses at which the agents inhibit platelet aggregation and thrombus formation by 50%, respectively. BT₂ values (mg kg⁻¹) are doses at which the agents double the control bleeding time (BT).

We also determined that *in vitro* effect of R-99224, a metabolite for CS-747, on ADP ($10 \mu\text{M}$)-induced platelet aggregation in rat PRP. Figure 6 shows typical changes in ADP induced aggregation tracing at different concentration of R-99224. Note that the *ex vivo* effects of CS-747 and *in vitro* effect of R-99224 on aggregation were similar (Figure 6). R-99224 (7.53 – $75.3 \mu\text{M}$) produced a concentration-related inhibition of platelet aggregation with an IC_{50} value of $44.9 \mu\text{M}$. In contrast, CS-747 (30 – $300 \mu\text{M}$) showed minimal effects on *in vitro* rat platelet aggregation, and the maximal inhibition at $300 \mu\text{M}$ was $9.0 \pm 2.7\%$ (the IC_{50} value $> 300 \mu\text{M}$, $n=5$).

Time course study The time course of the anti-aggregatory effect of CS-747 (1 – 10 mg kg^{-1}) after a single oral dosing were examined in comparison with clopidogrel (10 – 100 mg kg^{-1}). As shown in Figure 7A, a more than 80%

inhibition was observed in CS-747 (10 mg kg^{-1} , p.o.)-treated rats at 0.5 h after dosing. At this time, clopidogrel exhibited minimal inhibition even at the highest dose used (100 mg kg^{-1} , p.o.) (Figure 7B). The inhibitions of platelet aggregation by CS-747 (1 – 3 mg kg^{-1} , p.o.) and clopidogrel (10 – 30 mg kg^{-1} , p.o.) reached a plateau within 2 and 4 h post-dose, respectively. The inhibition of platelet aggregation by CS-747 was of long duration: a slight but significant inhibition was observed at 72 h post-dose in the CS-747-treated rats (Figure 7A). At 96 h post-dose there were no differences in platelet aggregation between the control and CS-747-treated groups. The long duration of antiaggregatory effects was also observed in the clopidogrel-treated animals (Figure 7B). The *ex vivo* effects of CS-747 on ADP (0.03 – $1 \mu\text{M}$)-induced platelet shape changes were determined at 4 h post-dose. Orally administered CS-747 (1 and 3 mg kg^{-1} , p.o.) did not significantly affect ADP-induced shape changes (data not shown).

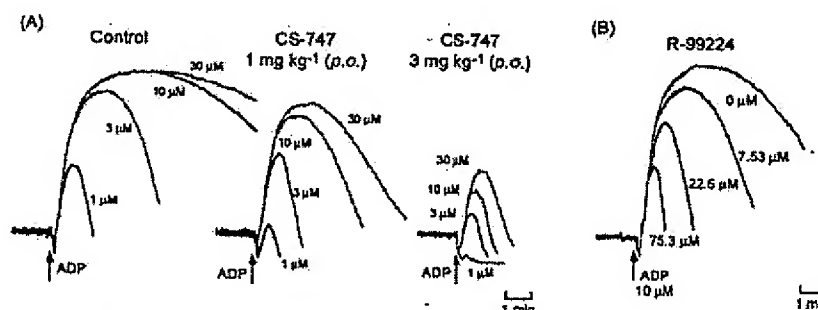


Figure 6 Representative aggregometer tracing showing the *ex vivo* effect of CS-747 (A) and *in vitro* effect of R-99224 (B) on ADP-induced platelet aggregation in rat PRP. CS-747 (1 and 3 mg kg^{-1} , p.o.) inhibited ADP (1 – $30 \mu\text{M}$)-induced platelet aggregation. R-99224 (7.53 – $75.3 \mu\text{M}$) inhibited $10 \mu\text{M}$ ADP-induced aggregation.

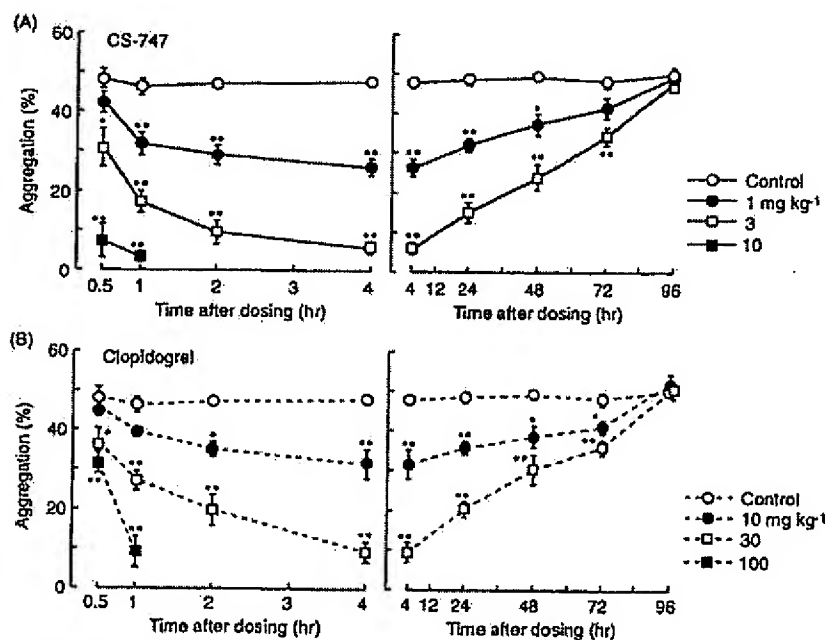


Figure 7 Time courses of the antiplatelet effects of CS-747 (A) and clopidogrel (B). *Ex vivo* platelet aggregation in PRP was measured at 0.5, 1, 2, 4, 24, 48, 72, and 96 h after the single oral administration of the agents. ADP at a concentration of $3 \mu\text{M}$ was used as an agonist. Results are presented as the mean \pm s.e. mean ($n=6$). * $P < 0.05$, ** $P < 0.01$ vs control (vehicle-treated group).

Antithrombotic effects in a rat arterio-venous shunt model

A rat model of arterio-venous shunt (Umetsu & Sanai, 1978; Sugidachi *et al.*, 1993) was used to examine the antithrombotic effects of CS-747 in comparison with those of clopidogrel and ticlopidine (Figure 8). After a 30 min circulation of blood through the canula, the weights of the thrombus formed on the silk thread were not different among the three control groups. In the CS-747 (0.1 – 3 mg kg⁻¹, p.o.) groups, thrombus formation was decreased in a dose-related manner, and the ED₅₀ value was estimated as 0.68 mg kg⁻¹. Clopidogrel (1 – 30 mg kg⁻¹, p.o.) also decreased thrombus weight in a dose-related manner, with an estimated ED₅₀ value of 6.2 mg kg⁻¹. Ticlopidine (10 – 300 mg kg⁻¹, p.o.) produced a significant decrease of thrombus weight, but the ED₅₀ value was not obtained (>300 mg kg⁻¹) since the maximum inhibition was less than 40%.

Bleeding time

Rat tail transection bleeding time (Dejana *et al.*, 1979; Sugidachi *et al.*, 1993) was measured to determine antihaemostatic effects of CS-747 in comparison to those of clopidogrel

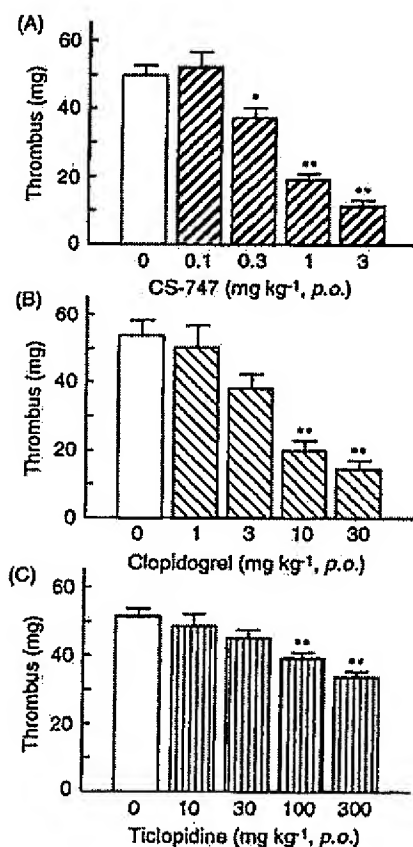


Figure 8 Effects of single administration of CS-747 (A), clopidogrel (B), and ticlopidine (C) on the arterio-venous shunt thrombosis model in rats. Blood circulation was started 4 h after the oral administration of the agents. Results are presented as the mean \pm s.e.mean ($n=6$). * $P<0.05$, ** $P<0.01$ vs control (vehicle-treated group).

and ticlopidine. The bleeding time averaged 446 ± 16 s ($n=7$) in the vehicle-treated control group. All the drugs tested prolonged bleeding time in a dose-related manner (Figure 9). The BT₂ values of CS-747, clopidogrel, and ticlopidine were 0.50 , 4.6 , and 130 mg kg⁻¹ (p.o.), respectively. To evaluate the efficacy and safety of these agents, the ratios of ED₅₀ for antiplatelet (ED_{50ADP}) and antithrombotic (ED_{50AV}) potencies to BT₂ values were calculated for each agent (Table 1).

Discussion

The results of this study demonstrated that CS-747 was a potent inhibitor of the G_i-linked P2T receptor *ex vivo*. Through this work, we showed that the inhibition of G_i-linked P2T receptor with CS-747 produced a specific and dose-related inhibition of *ex vivo* ADP-induced platelet aggregation which developed rapidly and lasted for a long period of time. CS-747 had potent antithrombotic effects in a rat model of arterial thrombosis, and antihaemostatic action in the rat bleeding-time assay. In contrast to these potent *ex vivo* and *in vivo* activities, CS-747 was inactive *in vitro*. R-99224, an *in vivo* metabolite of CS-747, however, produced a concentration-related inhibition of *in vitro* PRP aggregation. Thus, as in the case of clopidogrel and ticlopidine, *in vivo* activities of CS-747 are attributed to its active metabolite.

2-MeS-ADP is a stable agonist for P2Y₁ and G_i-linked P2T receptor (Kunapuli, 1998a,b). A single oral administration of CS-747 produced a significant but partial inhibition (about 43% inhibition at 10 mg kg⁻¹) of [³H]-2-MeS-ADP binding to platelets in the *ex vivo* study (Figure 2). Clopidogrel (100 mg kg⁻¹) also showed similar partial inhibition of [³H]-2-MeS-ADP binding. This agreed with the previous findings that clopidogrel and ticlopidine produced partial inhibition of [³H]-2-MeS-ADP binding to platelets (Savi *et al.*, 1994a; Gachet *et al.*, 1995), although the extent of maximal inhibition (%) was greater than our present study (approximately 70% vs 45%). This difference in maximal inhibition between the present study and the aforementioned studies may involve the use of different gender of rats or different technique of separation of the radioligand.

Recent studies have demonstrated that the platelet ADP receptor is not homogeneous. In addition to the ligand-gated ion channel P2X₁ receptor (MacKenzie *et al.*, 1996), two subclasses of G-protein-coupled ADP receptors have been proposed to exist on human platelet membranes: the G_i-linked

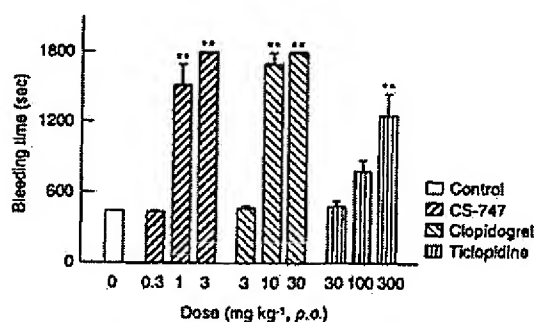


Figure 9 Effects of single administration of CS-747, clopidogrel, and ticlopidine on tail transection bleeding time in rats. Agents were orally administered to rats 4 h before the tail transection. Results are expressed as the mean \pm s.e.mean ($n=7$). ** $P<0.01$ vs control (vehicle-treated group).

P2Y₁ receptor and a G_i-linked P2T receptor (Fagura *et al.*, 1998; Daniel *et al.*, 1998; Santzen *et al.*, 1998). The G_i-linked P2T receptor has also been suggested to exist in platelets in the rat and the rabbit (Defreyne *et al.*, 1991; Savi *et al.*, 1996). Indeed, our present study confirmed that ADP lowers cyclic AMP concentration in rat platelets stimulated with PGE₁, an activator of adenylyl cyclase, and that CS-747 treatment inhibited this effect (Figure 3). In addition, CS-747 did not affect P2Y₁-mediated platelet shape change. These results suggest that metabolites of CS-747 selectively interfere with the G_i-linked P2T receptor on rat platelet membranes.

Treatment with CS-747 inhibited *ex vivo* washed platelet aggregation in response to ADP but not to thrombin (Figure 4). This is consistent with the hypothesis that the antiaggregatory action of CS-747 is due to its specific inhibition of the G_i-linked P2T receptor rather than its interference with the fibrinogen receptors. Nonetheless, collagen-induced aggregation was inhibited by CS-747, but this may be interpreted on the basis of previous studies demonstrating that platelet-derived ADP plays a major role in collagen-induced aggregation of rat platelets (Wey *et al.*, 1982; Tschopp & Zucker, 1972; Emms & Lewis, 1986). Indeed, apyrase, an ADP scavenger, has similar inhibitory profile on rat washed platelet aggregation. Hence, the inhibition of collagen-induced aggregation by CS-747 is most probably attributed to inhibition of the effects of ADP released from the dense granules of collagen-stimulated platelets.

The present study showed that CS-747 possesses an antiaggregatory efficacy approximately ten times more potent than that of clopidogrel (Figures 5 and 7). In sharp contrast, a single administration of ticlopidine caused minimal inhibition of platelet aggregation. This is in agreement with previous studies demonstrating clear antiaggregatory effects of ticlopidine only after repeated administrations in humans (McTavish *et al.*, 1990). In addition, the time course study showed that CS-747 (10 mg kg⁻¹) had a more rapid onset (<0.5 h) of antiaggregatory action than clopidogrel (Figure 7). The rapid onset of CS-747 was observed not only in rats but in various species including human (unpublished data). Although the precise mechanism responsible for the rapid onset of CS-747 remains to be elucidated, one possible explanation is that CS-747 may be more rapidly metabolized to its active metabolite *in vivo*. Ticlopidine and clopidogrel, which are inactive *in vitro*, reportedly must undergo hepatic metabolism to become active (Savi *et al.*, 1992), and it has been suggested that the metabolic activation of clopidogrel involves the cytochrome P450-1A subfamily (Savi *et al.*, 1994b). Further study is necessary to determine the metabolic pathways of CS-747, but the agent is speculated, based on its chemical structure, to generate active metabolites more readily than clopidogrel.

The time course study also showed that CS-747 had a long duration of antiaggregatory action. In fact, the durations of inhibition of platelet aggregation by CS-747 and clopidogrel in this study were comparable to the life span of circulating platelets in the rat (Caltaneo *et al.*, 1985; Jackson *et al.*, 1992). In addition, the antiaggregatory effects of CS-747 observed in washed platelets were not easily reversed by washing of platelets. Hence, it is likely that CS-747 inhibits platelet aggregation in an irreversible manner. Taken together, these results suggest that the active metabolite of CS-747 exerts antiaggregatory action through an irreversible modification of the G_i-linked P2T receptor on the membrane surface. Further studies are now underway to investigate the *in vitro* interaction between an active metabolite of CS-747 and platelet ADP receptors.

Several lines of evidence suggest that *in vivo* antiplatelet effects of thienopyridine derivatives are due to their active metabolite(s) as described above. But the active metabolites have not yet been reported so far. In contrast, Weber *et al.* (1999) reported that incubation of washed platelets with clopidogrel resulted in inhibition of ADP-induced *in vitro* platelet aggregation without hepatic bioactivation. This report also showed that clopidogrel did not show any inhibition of *in vitro* aggregation in PRP. Our present study confirmed that CS-747 did not show any inhibition of ADP-induced PRP aggregation. The present results, however, showed that R-99224, an *in vivo* metabolite for CS-747, inhibits ADP-induced *in vitro* platelet aggregation in the presence of plasma (Figure 6). Our preliminary results also have shown that R-99224 shows more potent antiaggregatory activity in washed platelets. To our knowledge, this is the first report that described the active metabolite for thienopyridine derivatives. Taken together, the inhibitory effects of CS-747 on platelet aggregation are, at least in part, likely to depend on its active metabolite, R-99224.

A rat model of arterio-venous shunt thrombosis has been extensively studied and used to examine antithrombotic effects of various agents (Maffrand *et al.*, 1988; Herbert *et al.*, 1996; Odawara *et al.*, 1996). In this model, CS-747 showed a potent antithrombotic efficacy that exceeds the potencies of clopidogrel and ticlopidine. The order of antithrombotic potencies among these agents was the same as the order of antiaggregatory potencies (Figure 8). In addition, CS-747 was devoid of *in vitro* and *ex vivo* anticoagulant or fibrinolytic activities (data not shown). Thus, the available data support the view that CS-747 exerts its antithrombotic effects by inhibiting the interaction between ADP and its receptors on the platelet membrane.

In this study, CS-747, clopidogrel, and ticlopidine all showed a significant and dose-related prolongation of the rat tail transection bleeding time (Figure 9). The order of the antithrombotic potency among these agents was the same as the order of antiplatelet and antithrombotic potencies. In addition, the ratios of antithrombotic dose (ED_{50AV}) and antiaggregatory dose (ED_{50AGG}) to antithrombotic dose (BT₂) for CS-747 and clopidogrel were approximately the same (Table 1). From these comparative studies, CS-747 and clopidogrel may have a similar ratio of benefit/bleeding risk. This might be clinically important, since clopidogrel is clinically available and its benefit/bleeding risk ratio has been identified in a large clinical study (CAPRIE Steering Committee, 1996). It should be noted that a single administration of ticlopidine produced a marked prolongation of bleeding time, whereas the same dose ranges of this agent exerted only mild to moderate antiplatelet and antithrombotic actions. Taken together, these results suggest that CS-747 is a potent antithrombotic and antiplatelet agent with relatively moderate antithrombotic potency, but this remains to be proven in future clinical studies.

The present study demonstrated that CS-747 is orally active and produces a potent antiplatelet and antithrombotic action with a rapid onset and long duration *via* platelet ADP receptors antagonism. CS-747 merits clinical evaluation in a variety of thrombotic diseases.

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Exhibit 22

The central role of the P_{2T} receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity

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Summary. Adenosine diphosphate (ADP) is an important platelet agonist and ADP released from platelet dense granules amplifies responses to other agonists. There are three known subtypes of ADP receptor on platelets: P_{2X}₁, P_{2Y}₁ and P_{2T} receptors. Sustained ADP-induced aggregation requires co-activation of P_{2Y}₁ and P_{2T} receptors. AR-C69931MX, a selective P_{2T} receptor antagonist and novel antithrombotic agent, was studied to characterize further the function of the P_{2T} receptor. The roles of the P_{2Y}₁ receptor and thromboxane A₂ were assessed using the selective P_{2Y}₁ antagonist A2P5P and aspirin respectively. Aggregation was measured by whole blood single-platelet counting and platelet-rich plasma turbidimetry, using hirudin anticoagulation. Dense granule release was estimated using [¹⁴C]-5-hydroxytryptamine (HT)-labelled platelets. Ca²⁺ mobilization, P-selectin expression, Annexin V

binding and microparticle formation were determined by flow cytometry. P_{2T} receptor activation amplified ADP-induced aggregation initiated by the P_{2Y}₁ receptor, as well as amplifying aggregation, secretion and procoagulant responses induced by other agonists, including U46619, thrombin receptor-activating peptide (TRAP) and collagen, independent of thromboxane A₂ synthesis, which played a more peripheral role. P_{2T} receptor activation sustained elevated cytosolic Ca²⁺ induced by other pathways. These studies indicate that the P_{2T} receptor plays a central role in amplifying platelet responses and demonstrate the clinical potential of P_{2T} receptor antagonists.

Keywords: platelet activation, P₂ receptors, anti-platelet drug, platelet secretion, calcium.

Adenosine diphosphate (ADP) activates platelets by binding to purinoceptors on the platelet surface and, in contrast to purinoceptors on other cell types, adenosine triphosphate (ATP) is a competitive antagonist for this process (Gachet *et al.*, 1996). Current evidence suggests that there are three types of ADP receptor on platelet surfaces, classified as P_{2X}₁, P_{2Y}₁ and P_{2T} (P_{2T}_{AC} or P_{2Y}_{ADP}) receptors (MacKenzie *et al.*, 1996; Daniel *et al.*, 1998; Fagura *et al.*, 1998; Geiger *et al.*, 1998; Jin *et al.*, 1998; Jantzen *et al.*, 1999; Leon *et al.*, 1999). Both the P_{2X}₁ and the P_{2Y}₁ receptors, but not the P_{2T} receptor, have been cloned (Jin *et al.*, 1998; Sun *et al.*, 1998). The P_{2T} receptor has been characterized pharmacologically, using selective antagonists, as the receptor linked, via Gi, to inhibition of adenylate cyclase, mediating a fall in the cyclic AMP level in response to ADP (Mills & Smith, 1972; Daniel *et al.*, 1998; Jin *et al.*, 1998; Savi *et al.*, 1998; Jantzen *et al.*, 1999). Studies of a patient with ADP receptor

deficiency support the concept that the P_{2T} receptor is a single, distinct receptor subtype (Leon *et al.*, 1999). Co-activation of both the P_{2Y}₁ and the P_{2T} receptors (G-protein coupled receptors) is required for platelet aggregation to occur, as detected by turbidimetry (Jin & Kunapuli, 1998). The P_{2X}₁ receptor (a ligand-gated ion channel), which is selectively activated by $\alpha\beta$ -methylene ATP, mediates rapid transient Ca²⁺ influx, but has not been found to contribute to platelet aggregation (MacKenzie *et al.*, 1996; Jin & Kunapuli, 1998; Kunapuli, 1998). The P_{2Y}₁ receptor activates phospholipase C (PLC), via Gq, and this accounts for most of the elevation in cytosolic Ca²⁺ induced by ADP, via formation of IP₃ and release of Ca²⁺ from intracellular stores (Daniel *et al.*, 1998; Leon *et al.*, 1999).

ADP may be released from platelet dense granules, where it is stored in high concentration, or erythrocytes and endothelial cells (Gachet *et al.*, 1996). ADP released by platelets stimulated by other agonists, such as thrombin or collagen, amplifies aggregation and secretion responses induced by these agonists (Cattaneo *et al.*, 1991, 1997; Colman *et al.*, 1994).

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Analogues of ATP that bind specifically to the P_{2T} receptor, including AR-C66096 and AR-C67085 (formerly ARL or FPL 66096 and ARL or FPL 67085 respectively) allow *in vitro* study of the function of the P_{2T} receptor (Humphries *et al*, 1994, 1995a, 1995b; Daniel *et al*, 1998; Fagura *et al*, 1998; Jin & Kunapuli, 1998; Jin *et al*, 1998). Blockade of the P_{2T} receptor with these agents abolishes the turbidimetric response to ADP (Jin & Kunapuli, 1998) and also renders aggregation induced by the thrombin receptor (PAR1)-activating peptide, TRAP, reversible (Trumel *et al*, 1999).

A more recent and related P_{2T} receptor antagonist, AR-C69931MX (Ingall *et al*, 1999), has been found to be a highly selective competitive antagonist at the P_{2T} receptor (unpublished observations) and is currently being developed as an intravenous antithrombotic agent. We have used this agent to study the role of the P_{2T} receptor in platelet aggregation and secretion, as well as its role in TRAP-induced procoagulant activity, as determined by platelet microparticle formation and Annexin V binding (Dachary-Prigent *et al*, 1993). We have related our findings to the role of thromboxane A₂ synthesis using aspirin. We have also assessed the effects of AR-C69931MX on agonist-induced rises in cytosolic Ca²⁺ to explore further the mechanism whereby P_{2T} receptor activation achieves its effects.

PATIENTS AND METHODS

Materials. ADP, epinephrine, U46619 (a thromboxane A₂ mimetic), TRAP (SFLLRNPNDKYEPF), aspirin, platelet-activating factor (PAF; dissolved in 0.25% w/v human serum albumin), EGTA, probenecid and the P2Y₂ receptor antagonist adenosine-2,5-biphosphate (A2P5P) were from Sigma. Collagen and dextrose buffer were from Nycomed. Saline was 0.9% NaCl from Baxter. AR-C69931MX [N-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-5'-adenylic acid, monoanhydride with dichloromethylenebisphosphonic acid] was provided by AstraZeneca R&D Charnwood, Loughborough, UK. Hirudin was recombinant desulphato-hirudin (Revasc), a gift from Novartis. 5-Hydroxytryptamine (5-HT) and [¹⁴C]-5-HT (1.85 MBq/ml) were from Amersham International. Chlorimipramine hydrochloride was from Novartis. Scintillation fluid was from BDH Laboratories Supplies. Fixative solution consisted of saline with 4.6 mmol/l sodium EDTA, 4.5 mmol/l Na₂HPO₄, 1.6 mmol/l KH₂PO₄ and 0.16% w/v formaldehyde, pH 7.4.

Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson), with light scatter and fluorescence channels set at logarithmic gain. Data were analysed using CELLQUEST software. Fluo-3 AM from Molecular Probes (Oregon, USA) was prepared as a 500 µmol/l solution in anhydrous dimethylsulphoxide (DMSO). CD62P-fluorescein isothiocyanate (FITC) antibody, mouse immunoglobulin (Ig)G-FITC and anti-CD42a-RPE fluorescent antibodies were from Serotec. Annexin V-FITC was from Alexis Biochemicals. HEPES/Tyroses (HT) buffer was 129 mmol/l NaCl, 8.9 mmol/l NaHCO₃, 2.8 mmol/l KCl, 0.8 mmol/l KH₂PO₄, 5.6 mmol/l dextrose and 10 mmol/l HEPES, pH 7.4. CaCl₂·6H₂O was from Fisons.

All concentrations given in subsequent sections are final concentrations in the reaction mixture.

Preparation of blood and platelet-rich plasma (PRP). Blood was obtained from healthy volunteers using a 19G needle and plastic syringe. All volunteers denied taking any non-steroidal anti-inflammatory drug in the previous 2 weeks. Aliquots of blood (9 ml) were transferred to polystyrene tubes containing anticoagulant. Hirudin (50 µg/ml; 900 anti-IIa units/ml) was mainly used as an anticoagulant to maintain physiological Ca²⁺ levels and avoid the artefactual enhancement of thromboxane A₂ synthesis that occurs in medium containing low Ca²⁺ levels, such as citrate (Mustard *et al*, 1975; Heptinstall & Mulley, 1977; Lages & Weiss, 1981; Packham *et al*, 1989). In some experiments, after an initial blood sample, healthy volunteers ingested aspirin 600 mg (UniChem) and further blood was venesected 2.5 h later for repeat analysis. Alternatively, *in vitro* aspirin 100 µmol/l was included with the anticoagulant. Trisodium citrate dihydrate (3.13% w/v; TCD) (1:9 TCD/blood) was used as an anticoagulant in some experiments for comparison with hirudin. For the release reaction experiments, [¹⁴C]-5-HT (0.521 µmol/l; 1.11 kBq/ml) was added to the anticoagulant (for uptake by platelets). For whole blood studies, the tubes were incubated at 37°C in a waterbath for a standard 30 min period before experimentation. PRP was prepared by centrifugation of blood at 180 g for 10 min and removal of the supernatant PRP. Platelet-poor plasma (PPP) was prepared by centrifugation of the remaining blood at 1500 g for 10 min and removal of the supernatant PPP. Platelet counts were performed on the PRP and the latter was diluted with PPP to obtain a final platelet count of 300 × 10⁹/l. PRP turbidimetry was performed using a BioData PAP-4 aggregometer.

Platelet aggregation in whole blood. Platelet aggregation studies were performed with whole blood using the single-platelet counting technique (Fox *et al*, 1982; Storey *et al*, 1998). Aliquots (460 µl) of blood were placed in polystyrene tubes with a magnetic stirrer bar and 20 µl of saline or AR-C69931MX or A2P5P was added. Samples were then stirred at 37°C (stirring speed 1000 r.p.m.) for 2 min. Blood (15 µl) was removed and fixed in 30 µl fixative to measure initial platelet count before addition of 20 µl agonist or saline. Aliquots (15 µl) of blood were removed at different time points after addition of agonists, as referred to in the Results section, and fixed in 30 µl fixative. Fixed samples were counted using the Ultra-Flo 100 platelet counter (Becton Dickinson) and percentage aggregation calculated as percentage loss of single platelets compared with baseline.

The following agonists were assessed: ADP (0.3–100 µmol/l), collagen (0.5–8 µg/ml), U46619 (0.3–3 µmol/l), epinephrine (10 µmol/l), 5-HT (3–30 µmol/l), PAF (0.1–1 µmol/l), TRAP (3–30 µmol/l) and α,β-methylene ATP (100 µmol/l). The effects of aspirin, alone or in combination with AR-C69931MX, were assessed for selected concentrations of agonists, as described in the Results section, using AR-C69931MX 100–1000 nmol/l to cover the estimated therapeutic levels to be achieved clinically.

P-selectin expression. Twenty microlitres of either saline or

AR-C69931MX 100, 300 or 1000 nmol/l was added to 460 µl whole blood incubated at 37°C. After 2 min, 20 µl agonist was added (see Results section) without continuous stirring and an aliquot was removed at 4 min for fixing, as above. Unstimulated samples were used for baseline measurement. Samples were incubated with a saturating concentration of anti-CD62P for 20 min, washed and resuspended in FACSflow. Non-specific binding was determined using mouse IgG-FITC antibody (an antibody of irrelevant specificity) in place of anti-CD62P. Samples were diluted in FACSflow and analysed by flow cytometry. A gate was applied to the platelet region and 2000 platelet events were collected. P-selectin expression was determined as the median fluorescence of the entire platelet population and results were expressed as an increase over baseline values.

[¹⁴C]-5-HT release. 5-HT release was determined by a previously validated method (Heptinstall *et al.*, 1980). Twenty microlitres of saline or AR-C69931MX and 20 µl chlorimipramine (1 µmol/l; to prevent 5-HT reuptake) were added to 440 µl blood containing [¹⁴C]-5-HT-labelled platelets and the sample was incubated at 37°C for 2 min. Agonist (20 µl) was then added and samples were stirred for 4 min. The reaction was terminated by placing on ice, as well as adding 50 µl of aspirin (1.26 mmol/l). Samples were centrifuged at 1500 *g* for 10 min and duplicate 50-µl samples of the supernatant plasma were placed in separate scintillation tubes that were then filled with scintillation fluid, capped and mixed. Sample radioactivity was counted in a scintillation counter. One hundred per cent release was estimated using duplicate 50-µl samples of a saline solution of [¹⁴C]-5-HT at the same concentration as that added to the whole blood sample, with adjustment of the results according to the packed cell volume of the blood sample to derive the initial plasma [¹⁴C]-5-HT concentration.

Microparticle formation and Annexin V binding. Microparticle formation and Annexin V binding induced by TRAP 20 µmol/l were measured in PRP with simultaneous turbidimetry. Aspirin (100 µmol/l) was included with the anticoagulant in some tubes. Saline or AR-C69931MX (20 µl) (10–1000 nmol/l) was added to 460 µl PRP and the samples were warmed for 1 min, then stirred for 1 min with assessment of baseline light transmittance. TRAP (20 µl) (20 µmol/l) was then added and change in light transmittance was assessed for 4 min. At 4 min, 15 µl PRP was removed and added to 30 µl HT buffer + 5 µl Annexin V-FITC + 5 µl CaCl₂ (100 mmol/l, final concentration 10 mmol/l) + 5 µl anti-CD42a-RPE. Anti-CD42a was used as a platelet membrane marker to gate out any artefact. Unstimulated PRP was used for a baseline measurement. Samples were incubated in the dark for 10 min at room temperature, then 1 ml HT buffer was added and samples were analysed by flow cytometry. Events were acquired for 10 s. Microparticle formation was assessed by setting the marker for forward scatter to include 1% of events with the lowest forward scatter for the baseline sample. Annexin V binding was assessed by setting the marker for Annexin V-positive events to include 1% events for the baseline sample. These markers were then applied to other samples. The baseline values were subtracted from the results for stimulated samples.

Ca²⁺ measurements. Aliquots (2 ml) of PRP were incubated with fluo-3 AM (5 µmol/l) in the presence of probenecid (2.5 mmol/l) for 30 min at 37°C. Samples were then maintained at room temperature. Aliquots (10 µl) of labelled PRP were added to 1 ml HT buffer at 37°C containing CaCl₂·6H₂O (1 mmol/l) or EGTA (100 µmol/l), giving a 1:100 dilution of PRP. Aliquots (250 µl) of diluted PRP were then applied to the flow cytometer to measure the baseline fluorescence. A further 480-µl aliquot was added to a tube containing ADP (0.3 µmol/l), TRAP (20 µmol/l) or a combination of both agonists (to mimic the physiological effects of released ADP on the action of TRAP in undiluted PRP). The fluorescence of the sample was then measured after 5, 15, 30, 60 and 120 s. The median fluorescence intensity was determined for each time point.

Statistical analysis. Data were analysed using ANOVA for repeated measures on SPSS software and significance assigned to *P*-values less than 0.05. Data are expressed as means ± SEM. Correlation was measured using Excel 97 polynomial trend analysis.

RESULTS

Roles of the purinoceptor subtypes in ADP-induced platelet aggregation

The roles of the P2X₁, P2Y₁ and P2_T receptors in ADP-induced aggregation were studied using whole blood single-platelet counting, which is sensitive to microaggregation, assessing the aggregation response to the selective P2X₁ agonist α,β-methylene ATP and inhibition of ADP-induced aggregation by the selective P2Y₁ and P2_T antagonists A2P5P and AR-C69931MX respectively. α,β-Methylene ATP, at concentrations up to 100 µmol/l, did not induce even a transient aggregation response (including at 10 s after addition of this agonist), indicating that P2X₁ receptor activation alone is not sufficient to induce microaggregation. This is consistent with previous reports that P2X₁ receptor activation does not cause aggregation (Savi *et al.*, 1997; Kunapuli, 1998). Typical effects of the P2Y₁ and P2_T antagonists are represented by a comprehensive study of the time-course of aggregation induced by ADP 0.3 µmol/l, assessing the effects of a wide range of concentrations of AR-C69931MX and A2P5P (Fig 1). AR-C69931MX, at increasing concentrations, reduced the extent of aggregation and reduced the time to peak aggregation, indicating that the P2_T receptor sustains and amplifies ADP-induced aggregation. There was a residual early aggregation peak at 10 s that was resistant to increasing concentrations of AR-C69931MX, despite the low concentration of ADP used. Further studies revealed that each donor had a threshold ADP concentration at which increasing concentrations of AR-C69931MX (up to an excessive concentration of 100 µmol/l) failed to abolish this early aggregation response, indicating that P2Y₁ receptor activation alone is capable of producing a transient aggregation response. In contrast, A2P5P tended to flatten the aggregation response consistent with a progressive reduction in the proportion of aggregating platelets with increasing concentrations of A2P5P. These observations are consistent with the P2Y₁

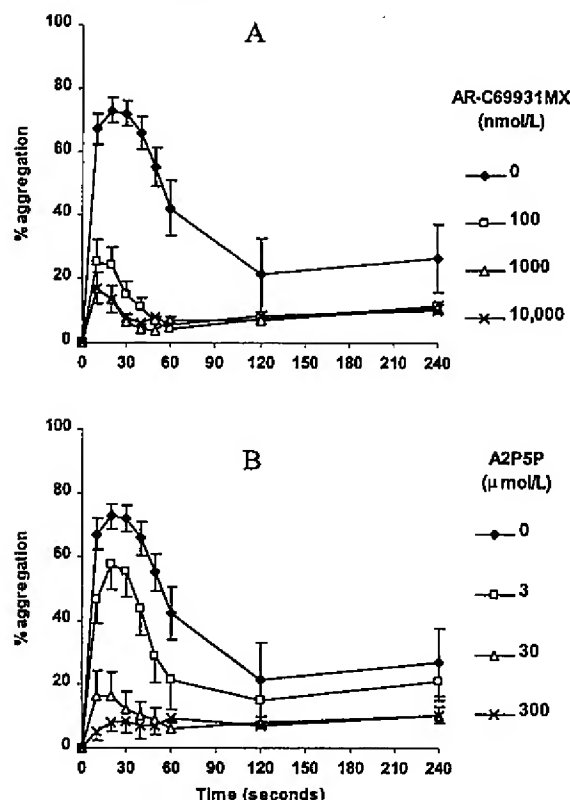


Fig 1. Inhibition of platelet aggregation induced by $0.3 \mu\text{mol/l}$ ADP by (A) the P_{2T} antagonist AR-C69931MX and (B) the P_{2Y_1} antagonist A2P5P, as assessed by whole blood single-platelet counting, illustrating different effects of the antagonists on the initial aggregation response ($n = 6$). Note that in (A) the effects of 1000 and 10 000 nmol/l AR-C69931MX are superimposed, indicating saturation of the effects of P_{2T} receptor blockade. Results are means \pm SEM.

receptor initiating ADP-induced aggregation and the P_{2T} receptor amplifying and sustaining this response, as suggested previously (Hechler *et al*, 1998; Jarvis *et al*, 2000). Simultaneous PRP turbidimetry and single-platelet counting, as described previously (Storey *et al*, 1998), showed that AR-C69931MX could abolish the turbidimetric response to ADP concentrations up to $10 \mu\text{mol/l}$, but there remained a transient microaggregation response (mediated by the P_{2Y_1} receptor) that was detected by single-platelet counting but not by turbidimetry (data not shown).

Both AR-C69931MX and A2P5P induced disaggregation of ADP-induced aggregates (Fig 2), indicating that continuous P_{2T} and P_{2Y_1} receptor occupancy by ADP are required for sustained ADP-induced aggregation.

Effects of AR-C69931MX and aspirin on platelet aggregation induced by different agonists

The effects of AR-C69931MX and aspirin were compared to determine the relative contributions of P_{2T} receptor activation and thromboxane A_2 synthesis to platelet responses to different agonists, as well as to develop further a rationale

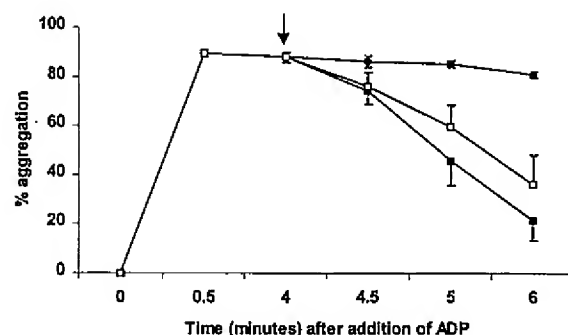


Fig 2. Disaggregation of platelet aggregates induced by ADP $10 \mu\text{mol/l}$ by addition of AR-C69931MX 1000 nmol/l (closed squares) and A2P5P $300 \mu\text{mol/l}$ (open squares) as indicated by arrow, compared with saline control (closed circles) after 4 min aggregation in whole blood, as assessed by single-platelet counting ($n = 6$). Results are means \pm SEM.

for the therapeutic use of AR-C69931MX. The effects of *in vitro* aspirin on aggregation and secretion were identical to the *ex vivo* effects of aspirin, therefore only the latter are illustrated.

Figure 3A illustrates the inhibition by aspirin and AR-C69931MX of aggregation induced by different agonists in hirudinized whole blood, determined at 4 min after addition of the agonist. AR-C69931MX inhibited ADP-induced aggregation in a concentration-dependent manner, whereas aspirin had no effect. Inhibition by AR-C69931MX of aggregation induced by agonists other than ADP was generally more pronounced at lower concentrations of agonist, as demonstrated by the lower concentrations of TRAP and collagen. Although there was not significant inhibition by AR-C69931MX of aggregation induced by U46619 $1 \mu\text{mol/l}$, in other experiments aggregation induced by U46619 $0.3 \mu\text{mol/l}$ was inhibited by AR-C69931MX 100 nmol/l (26.1% vs. 7.0% ; $P < 0.01$). It was therefore clearly demonstrated that AR-C69931MX had a broad-spectrum inhibitory effect on aggregation induced by all agonists, reflecting inhibition of P_{2T} receptor activation by released ADP. Only when the concentration of some agonists was raised to a level sufficient to cause maximal aggregation in the absence of the contributory effects of P_{2T} receptor activation by released ADP did AR-C69931MX fail to show a significant inhibitory effect on aggregation. The effect of AR-C69931MX on PAF-induced aggregation was primarily to render aggregation substantially more reversible: maximal aggregation induced by $1 \mu\text{mol/l}$ PAF occurred at 30 s and 100 nmol/l AR-C69931MX only reduced this from $83.9 \pm 1.8\%$ to $77.3 \pm 2.5\%$, whereas at 4 min, after disaggregation had occurred, aggregation was reduced from $54.4 \pm 29.1\%$ to $8.5 \pm 5.7\%$ ($P < 0.01$). This suggests that P_{2T} receptor occupancy by ADP is necessary for the effects of PAF to be sustained, as well as those of ADP. Aggregation induced by low concentrations of collagen and TRAP was also rendered more reversible by AR-C69931MX.

Aspirin treatment yielded significant inhibition of aggregation induced by the lower concentration of collagen

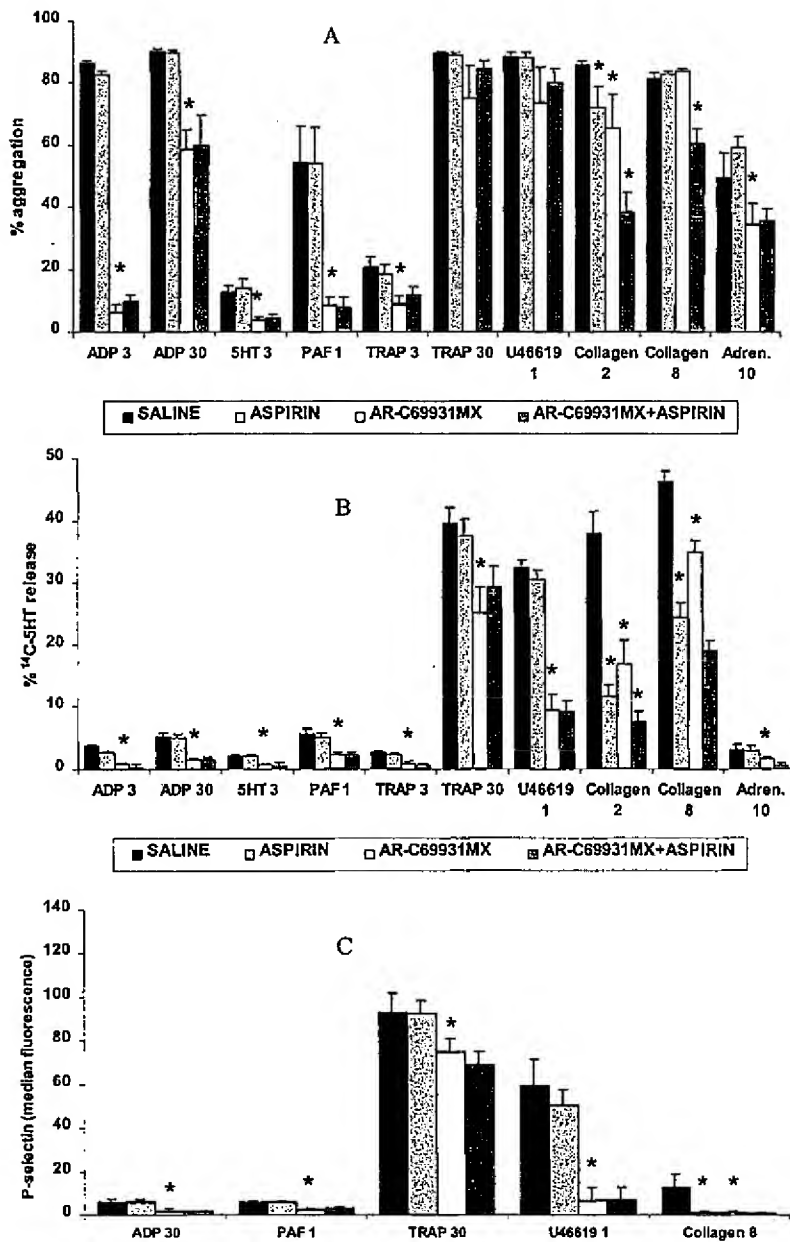


Fig 3. Mean (A) percentage platelet aggregation (whole blood single-platelet counting), (B) percentage [^{14}C]-5-HT release and (C) P-selectin expression (median fluorescence units) before and after ingestion of 600 mg aspirin with/without *in vitro* addition of 100 nmol/l AR-C69931MX 2 min before agonist ($n = 6$). Concentrations of agonists are as indicated either in μ mol/l or, for collagen, in μ g/ml. Results are means \pm SEM. * $P < 0.05$ for effect of aspirin, effect of AR-C69931MX and interaction between AR-C69931MX and aspirin.

(2 μ g/ml), but no significant inhibition of aggregation induced by ADP, 5-HT, PAF, TRAP, U46619 (at any concentration), 8 μ g/ml collagen or epinephrine. The combination of aspirin and AR-C69931MX yielded synergistic inhibition of aggregation induced by collagen.

[^{14}C]-5-HT release

The effects of aspirin and AR-C69931MX in whole blood on [^{14}C]-5-HT release (reflecting dense granule release) are shown in Fig 3B. Again, AR-C69931MX inhibited 5-HT release induced by all the agonists, markedly so in the case of U46619, demonstrating a central role for the P_{2T} receptor in the amplification of platelet dense granule secretion.

Inhibition of 5-HT release by AR-C69931MX was evident even when there was no inhibition of aggregation induced by stronger agonist stimulation, reflecting the fact that greater platelet activation is required for maximal secretion than for maximal aggregation. Aspirin only inhibited 5-HT release induced by collagen and had no effect on 5-HT release induced by the other agonists. When citrate, rather than hirudin, was used as the anticoagulant, ADP-induced 5-HT release was enhanced, as previously described (Mustard *et al.*, 1975; Heptinstall & Mulley, 1977; Lages & Weiss, 1981; Packham *et al.*, 1989), and this was then significantly inhibited by both aspirin and AR-C69931MX (data not shown).

P-selectin expression

The effects of AR-C69931MX and aspirin in whole blood on P-selectin expression (reflecting alpha-granule release) are shown in Fig 3C. 5-HT and epinephrine did not induce significant P-selectin expression (data not shown). The lower concentrations of TRAP and collagen were not studied. Again, AR-C69931MX inhibited P-selectin expression induced by all the agonists that produced a significant response and, again, there was dramatic inhibition by AR-C69931MX of the intensity of secretion induced by U46619.

Aspirin inhibited P-selectin expression induced by collagen, but had no effect on P-selectin expression induced by ADP, PAF, TRAP or U46619. When citrate was used as the anticoagulant, aspirin also weakly inhibited TRAP-induced P-selectin expression ($P < 0.05$).

Effect of concentration of AR-C69931MX

AR-C69931MX inhibited ADP-induced aggregation and secretion in a concentration-dependent manner. Whole blood concentrations of AR-C69931MX up to 100 nmol/l inhibited aggregation and secretion induced by other agonists in a concentration-dependent manner, but there was little or no significant difference between the effects of 100 nmol/l AR-C69931MX and 1000 nmol/l AR-C69931MX on aggregation, P-selectin expression or 5-HT release induced by agonists other than ADP, indicating a potent yet saturable effect of AR-C69931MX on responses to these agonists (data not shown). The most significant difference was seen for collagen-induced responses, e.g. [14 C]-5-HT release induced by 2 μ g/ml collagen was reduced from $34.0 \pm 4.5\%$ to $17.1 \pm 2.3\%$ by 100 nmol/l AR-C69931MX and to $14.3 \pm 2.8\%$ by 1000 nmol/l AR-C69931MX (effect of AR-C69931MX concentration: $P = 0.001$).

Microparticle formation and Annexin V binding

The effects of aspirin and AR-C69931MX on microparticle formation and Annexin V binding induced by TRAP 20 μ mol/l in PRP were assessed (Fig 4), with simultaneous turbidimetry (not shown). AR-C69931MX rendered TRAP-induced aggregation reversible, as shown for AR-C66096 (Trumel *et al*, 1999), and inhibited microparticle formation and Annexin V binding in a concentration-dependent manner ($P < 0.001$). Concentrations of > 100 nmol/l AR-C69931MX completely reversed aggregation and abolished microparticle formation and Annexin V binding. There was a strong correlation between reversibility of TRAP-induced aggregation in the presence of AR-C69931MX and inhibition of these procoagulant responses ($R^2 = 0.894$, inhibition of Annexin V binding vs. inhibition of aggregation at 4 min). Aspirin, on the other hand, showed no significant effect on aggregation, microparticle formation or Annexin V binding.

Ca²⁺ measurements

Data are presented as mean percentages of the maximum fluorescence obtained with the combination of ADP and TRAP as agonists (Fig 5). ADP evoked similar increases in

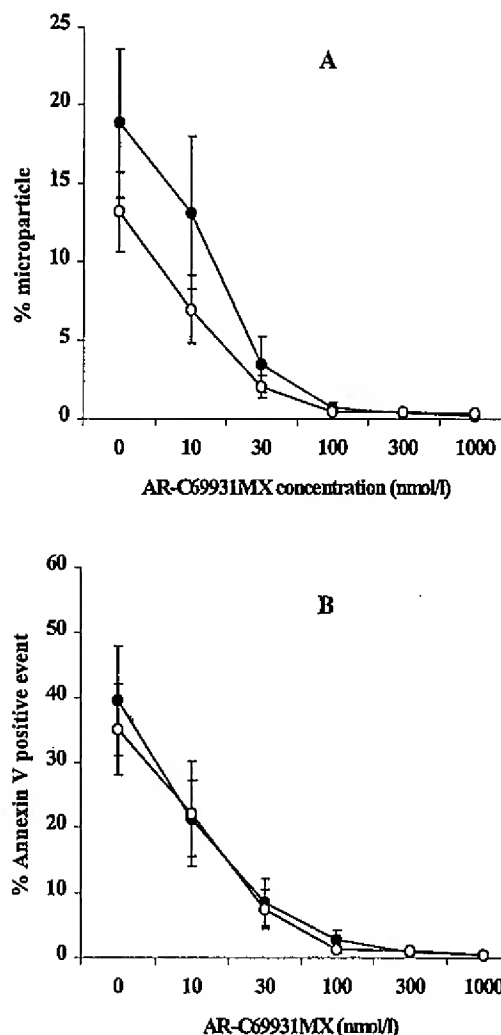


Fig 4. Inhibition of procoagulant activity by AR-C69931MX assessed by (A) microparticle formation and (B) Annexin V binding induced by 20 μ mol/l TRAP after 4 min aggregation in platelet-rich plasma ($n = 6$). Closed circles represent control samples and open circles represent samples that have been incubated with 100 μ mol/l aspirin. Results are means \pm SEM.

cytosolic Ca^{2+} in the presence and absence of extracellular Ca^{2+} and these were abolished by the $P2Y_1$ antagonist A2P5P, as previously described (Jin *et al*, 1998). AR-C69931MX had no significant effect on the initial rise in intracellular Ca^{2+} but accelerated the decay of Ca^{2+} , both in the presence and absence of extracellular Ca^{2+} , indicating that the P_{2T} receptor sustains elevated Ca^{2+} induced by $P2Y_1$ receptor activation. TRAP induced only slightly greater increases in peak cytosolic Ca^{2+} than ADP with a more sustained response, and these increases were not significantly affected by either A2P5P or AR-C69931MX. However, analyses were performed on PRP samples that had been diluted 100-fold, and this dilution would be expected to remove most of the effects of released ADP. Therefore, to mimic the physiological effects of TRAP, the combination of

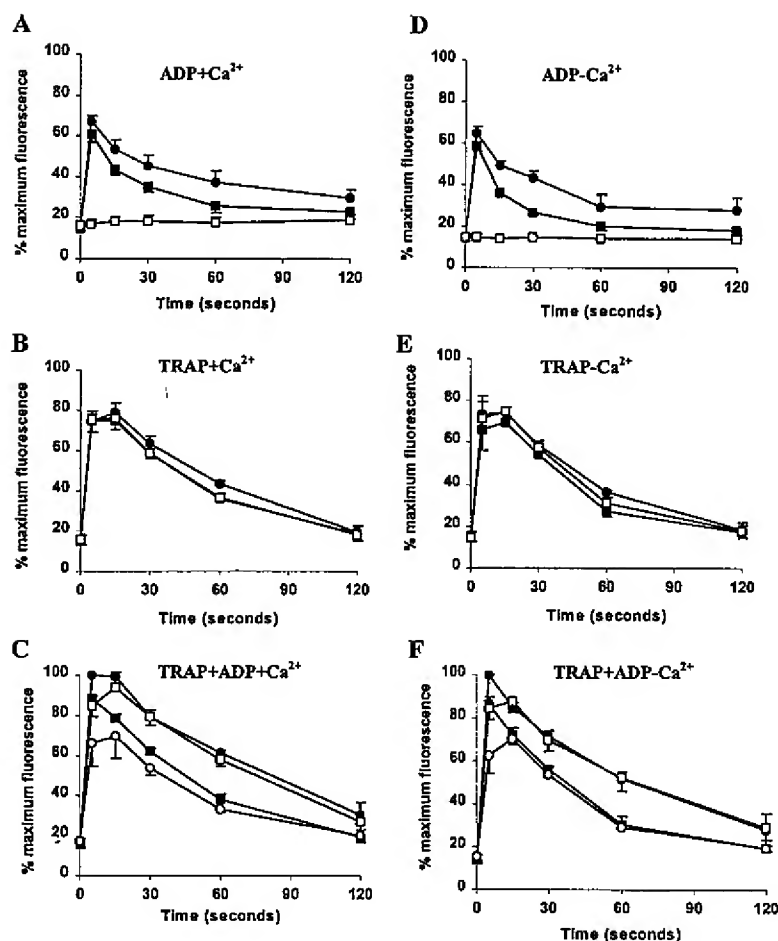


Fig 5. Cytosolic Ca^{2+} measurements using fluo-3 AM-labelled platelets and $0.3 \mu\text{mol/l}$ ADP (A and D), $20 \mu\text{mol/l}$ TRAP (B and E) or both agonists (C and F) in the presence (A–C) or absence (D–F) of extracellular Ca^{2+} . The effects of 1000 nmol/l AR-C69931MX (closed squares) and $300 \mu\text{mol/l}$ A2P5P (open squares) were assessed in comparison with saline control (closed circles), as well as the combination of both antagonists (open circles, C and F only). Results are means \pm SEM of three experiments and are expressed as a percentage of the maximum fluorescence obtained with ADP + TRAP. Maximum fluorescence was 87.9 ± 13.0 and 95.3 ± 15.1 fluorescence units in the presence and absence of extracellular calcium respectively.

TRAP and ADP was studied. ADP added substantially to the initial increase in cytosolic Ca^{2+} induced by TRAP and led to greater Ca^{2+} levels over the 2-min period compared with TRAP alone. A2P5P attenuated this additional increase in peak Ca^{2+} at 5 s, but failed to prevent a subsequent rise in Ca^{2+} to levels achieved in the absence of A2P5P and had no effect on the slope of decay. In contrast, AR-C69931MX both attenuated the 5-s peak Ca^{2+} level and accelerated the decay of cytosolic Ca^{2+} in an identical fashion to its effects in the studies of ADP alone. The combination of A2P5P and AR-C69931MX restored the response to that seen with TRAP alone. Thus, P_{2T} receptor activation sustains elevated Ca^{2+} levels induced by TRAP as well as by P_{2Y_1} receptor activation. Removal of extracellular Ca^{2+} by EGTA slightly enhanced the rate of decay of cytosolic Ca^{2+} , but had no effect on the inhibitory profiles of A2P5P or AR-C69931MX.

DISCUSSION

Central role of the P_{2T} receptor

These studies demonstrate that the P_{2T} receptor plays a central role in the amplification of platelet aggregation, secretion and procoagulant activity induced by the whole

range of natural agonists or their mimetics. We have also demonstrated an association between this role and the effects of the P_{2T} receptor on cytosolic Ca^{2+} levels that have been elevated by other receptor pathways. Considerable evidence has accumulated recently to support these findings: congenital deficiency of the P_{2T} receptor is associated with impaired dense granule release in response to ADP, U46619 and collagen (Nurden *et al.*, 1995; Cattaneo *et al.*, 1997; Leon *et al.*, 1999); the P_{2T} receptor antagonist AR-C66096 inhibits processes associated with platelet secretion, namely P-selectin expression induced by some radiographic contrast media (Heptinstall *et al.*, 1998) and aggregation and secretion induced by sera from patients with heparin-induced thrombocytopenia (Polgar *et al.*, 1998); P_{2T} receptor activation has been previously shown to be necessary for sustained aggregation induced by TRAP, via stimulation of PI 3-kinase (Trumel *et al.*, 1999); and several studies have shown that thienopyridines, which act on the P_{2T} receptor, inhibit dense granule release induced by some agonists, including low concentrations of thrombin and platelet-activating factor, but not others, including collagen (Hardisty *et al.*, 1990; Cattaneo *et al.*, 1991; Heptinstall *et al.*, 1995). The dramatic effects of AR-C69931MX on some responses, such as the secretion

response to the thromboxane A₂ mimetic U46619 or the procoagulant response to TRAP, show how crucial co-activation of the P_{2T} receptor by released ADP is for a full response to these agonists. Thus, ADP, although a relatively weak agonist by itself, is a powerful co-factor in the responses to stronger agonists via activation of the P_{2T} receptor.

Our studies of cytosolic Ca²⁺ show how the P_{2T} receptor does not contribute to the initial rise in Ca²⁺ induced by ADP, which is known to be mediated by the P_{2Y₁} and, to a lesser extent, P_{2X₁} receptors. Studies in the presence of EGTA, which chelates extracellular Ca²⁺ and therefore abolishes the effect of P_{2X₁} receptor activation, showed little difference from those studies performed in the presence of Ca²⁺, indicating no significant contribution of the P_{2X₁} receptor activation to the results obtained. We have shown how the P_{2T} receptor sustains the elevated cytosolic Ca²⁺ induced not only by P_{2Y₁} receptor activation but also by PAR1 activation with TRAP. Studies of the effect of clopidogrel on Ca²⁺ measurements using fura-2-loaded platelets showed no effect either on cytosolic Ca²⁺ rises induced by ADP or on the subsequent decay of Ca²⁺ (Hechler *et al.*, 1998), reflecting either differences in methodology or incomplete P_{2T} receptor blockade by clopidogrel. Similar to our findings, activation of the α_{2A}-adrenergic receptor, which like the P_{2T} receptor is linked to Gi and inhibition of adenylate cyclase, has been shown to potentiate Ca²⁺ release in response to stimuli such as thrombin (Keularts *et al.*, 2000). The close association between cytosolic Ca²⁺ and platelet aggregation and secretion is well established (Hawiger *et al.*, 1994). There is also close association between cytosolic Ca²⁺ and procoagulant changes in transbilayer lipid distributions as aminophospholipid translocase, which maintains phospholipid asymmetry, is inhibited by micromolar concentrations of Ca²⁺ and lipid scramblase activity is Ca²⁺ dependent (Zwaal & Schroit, 1997). Thus, maintenance of cytosolic Ca²⁺ levels by P_{2T} receptor activation may at least partly explain the effects of AR-C69931MX on platelet aggregation, secretion and procoagulant activity. The signalling pathways whereby P_{2T} receptor activation achieves these amplification effects are currently unclear and the relevance of cyclic AMP has not been established.

Roles of the P_{2T} and P_{2Y₁} receptors in ADP-induced aggregation

The effects of specific P_{2T} and P_{2Y₁} antagonists on platelet microaggregation in response to ADP have not been previously demonstrated and these studies add further weight to previous observations on the relative contribution of the P_{2T} and P_{2Y₁} receptors. Previously, it has been stated that both receptors are required for aggregation as assessed by turbidimetry, a measure of macroaggregation (Jin & Kunapuli, 1998). Using the more sensitive measure of single-platelet counting, we have shown that microaggregation can occur when the P_{2T} receptor is blocked; however, this aggregation is not sustained for sufficient time to allow the formation of macroaggregates, explaining why macroaggregation is not detected by turbidimetry. In contrast, P_{2Y₁} receptor blockade is sufficient to abolish aggregation.

Our findings concur with previous work suggesting that the P_{2Y₁} receptor initiates platelet aggregation, with the P_{2T} receptor playing a synergistic role (Hechler *et al.*, 1998; Jarvis *et al.*, 2000). The rapid disaggregatory effects of AR-C69931MX and A2P5P demonstrate how ADP-induced aggregation requires continued occupancy of both the P_{2T} and the P_{2Y₁} receptors by ADP and suggests that displacement of ADP from either receptor leads to deactivation of GPIIb/IIIa complexes and release of fibrinogen. The mechanism by which this occurs warrants further study.

Limitations of aspirin therapy and the potential role of P_{2T} receptor antagonists

These studies demonstrate the limitations of aspirin therapy with regard to inhibition of platelet responses when studied at physiological divalent cation levels. Although it is clear that thromboxane A₂ is an important agonist in thrombotic disease and that inhibiting thromboxane A₂ production will reduce secretion and aggregation induced by this agonist (as mimicked in these studies by U46619), it is also clear that the majority of platelet responses remain intact despite aspirin treatment. Previous studies of aspirin have shown how *in vitro* lowering of the divalent cation level with citrate anticoagulation facilitates thromboxane A₂ production, particularly in response to ADP (Mustard *et al.*, 1975; Heptinstall & Mulley, 1977; Lages & Weiss, 1981; Packham *et al.*, 1989). This explains why the effects of aspirin appear more limited when physiological divalent cation levels are maintained *in vitro* by using a direct thrombin inhibitor such as hirudin. Our studies show how P_{2T} receptor antagonism yields much greater inhibition of platelet function than the relatively weak effects of aspirin. However, the additive effects of aspirin and AR-C69931MX on collagen-induced responses illustrate the rationale for using the combination of these two agents for anti-platelet therapy.

The effects of AR-C69931MX on platelet secretion and procoagulant activity are important with regard to its potential role in the management of arterial thrombosis. Secreted ADP and 5-HT play an important role in recruiting platelets to arterial thrombosis, and 5-HT contributes to vasoconstriction and reduced arterial flow (Willerson *et al.*, 1989). Procoagulant activity of activated platelets contributes to local thrombin generation and thrombin is another important soluble agonist that recruits platelets to arterial thrombi, as well as generating fibrin (Badimon & Badimon, 1996). Platelet microparticles may also contribute to further activation of platelets, as well as to activation of monocytes and endothelial cells (Barry & FitzGerald, 1999). Thus, the effects of AR-C69931MX *in vitro* appear to support strongly further investigation of its use as an antithrombotic agent for managing arterial thrombosis. The first phase II study of AR-C69931MX in patients with acute coronary syndromes demonstrated that an infusion dose of 4 µg/kg/min achieves a mean plasma level equivalent to a plasma AR-C69931MX concentration of 484 nmol/l (Storey *et al.*, 1999), showing that the *in vitro* studies presented here are representative of the likely *in vivo* effects of this agent.

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Exhibit 23

Molecular Mechanism of Thromboxane A₂-induced Platelet Aggregation

ESSENTIAL ROLE FOR P2T_{AC} and α_{2A} RECEPTORS*

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Thromboxane A₂ is a positive feedback lipid mediator produced following platelet activation. The G_q-coupled thromboxane A₂ receptor subtype, TP α , and G_i-coupled TP β subtype have been shown in human platelets. ADP-induced platelet aggregation requires concomitant signaling from two P2 receptor subtypes, P2Y₁ and P2T_{AC}, coupled to G_q and G_i, respectively. We investigated whether the stable thromboxane A₂ mimetic, (15S)-hydroxy-9,11-epoxymethanoprostanoic acid (U46619), also causes platelet aggregation by concomitant signaling through G_q and G_i, through co-activation of TP α and TP β receptor subtypes. Here we report that secretion blockade with Ro 31-8220, a protein kinase C inhibitor, completely inhibited U46619-induced, but not ADP- or thrombin-induced, platelet aggregation. Ro 31-8220 had no effect on U46619-induced intracellular calcium mobilization or platelet shape change. Furthermore, U46619-induced intracellular calcium mobilization and shape change were unaffected by A3P5P, a P2Y₁ receptor-selective antagonist, and/or cyproheptadine, a 5-hydroxytryptamine subtype 2A receptor antagonist. Either Ro 31-8220 or AR-C66096, a P2T_{AC} receptor selective antagonist, abolished U46619-induced inhibition of adenylyl cyclase. In addition, AR-C66096 drastically inhibited U46619-mediated platelet aggregation, which was further inhibited by yohimbine, an α_{2A} -adrenergic receptor antagonist. Furthermore, inhibition of U46619-induced platelet aggregation by Ro 31-8220 was relieved by activation of the G_i pathway by selective activation of either the P2T_{AC} receptor or the α_{2A} -adrenergic receptor. We conclude that whereas thromboxane A₂ causes intracellular calcium mobilization and shape change independently, thromboxane A₂-induced inhibition of adenylyl cyclase and platelet aggregation depends exclusively upon secretion of other agonists that stimulate G_i-coupled receptors.

Upon exposure to activating agonists (e.g. thrombin, ADP, and collagen), platelets liberate arachidonic acid stored as phospholipid in the platelet plasma membrane that is con-

verted into thromboxane A₂ by sequential oxygenation of arachidonic acid by cyclooxygenase and thromboxane A₂ synthase (1). The released thromboxane A₂ acts as a positive feedback mediator in the activation and recruitment of more platelets to the primary hemostatic plug (2). Thromboxane A₂ exerts its actions via specific G protein-coupled receptors and has been described as either a potent platelet agonist (2, 3) or as a weak agonist with an important role in amplifying the response of platelets to more potent agonists (4).

Pharmacological studies indicate the presence of two potential thromboxane A₂ receptor (TP receptor)¹ subtypes on human platelets (5, 6). The TP receptor gene has been cloned and encodes two subtypes of the TP receptor that result from alternative splicing of the primary transcript (7). The subtypes share the identical first 293 amino acids but possess different carboxyl-terminal domains. A complete cDNA of the 343 amino acid TP α isoform was isolated from both a placental cDNA library and human megakaryocytic leukemia cells (8, 9) and a chronic myelogenous leukemia cell line (10). A cDNA for the 407-amino acid TP β subtype was cloned from a vascular endothelial library (11, 12). Both the TP α and TP β subtypes mediate the stimulation of phospholipase C and an increase in intracellular concentrations of inositol 1,4,5-triphosphate and diacylglycerol. The formation of inositol 1,4,5-triphosphate induces an increase in the cytosolic concentration of Ca²⁺, whereas the release of diacylglycerol activates PKC (13–16). In transfected cell lines the two subtypes were shown to oppositely regulate levels of cAMP. The TP α receptor stimulated cAMP formation in contrast to the TP β receptor that inhibited the level of intracellular cAMP (15). Pertussis toxin was shown to block TP β receptor-mediated inhibition of adenylyl cyclase; however, its effect on phospholipase C activation was not determined (15). By using isoform-specific antibodies Habib *et al.* (17) only detected the presence of the TP α receptor in human platelets. Hirata *et al.* (15) have shown the presence of mRNA encoding both TP α and TP β subtypes in platelets using reverse transcriptase-polymerase chain reaction.

ADP-induced platelet aggregation results from concomitant signaling through the P2Y₁ and P2T_{AC} receptors that couple to G_q and G_i, respectively (18–21). Thrombin has been shown to activate both G_q- and G_i-signaling cascades (22, 23). Contrary

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¹ The abbreviations used are: TP receptor, thromboxane A₂ receptor; U46619, (15S)-hydroxy-9,11-epoxymethanoprostanoic acid also known as 9,11-dideoxy-9 α ,11 α -methanoprostaglandin F_{2 α} ; P2T_{AC}, platelet ADP receptor coupled to inhibition of adenylyl cyclase; P2Y₁, platelet ADP receptor coupled to stimulation of phospholipase C; G_i, heterotrimeric GTP-binding protein which inhibits adenylyl cyclase; G_q, heterotrimeric GTP-binding protein that stimulates phospholipase C; 5-HT, 5-hydroxytryptamine; A3P5P, adenosine-3'-phosphate-5'-phosphate; PRP, platelet-rich plasma; PKC, protein kinase C; TxA₂, thromboxane A₂.

to previous studies, we have demonstrated that epinephrine and serotonin activating only G_i or G_q pathways, respectively, are not true platelet-aggregating agents (18). Offermanns *et al.* (24) have provided evidence showing that U46619 couples to G_q . Since thromboxane A_2 couples to two TP receptor subtypes and $TP\beta$ has been shown to inhibit adenylyl cyclase, we investigated whether U46619 (a stable thromboxane A_2 analog) also causes platelet aggregation by co-activation of $TP\alpha$ and $TP\beta$ receptor subtypes coupled to G_q and G_i , respectively.

We report here that U46619 causes intracellular calcium mobilization and shape change in human platelets independently of secretion. However, TxA_2 -induced platelet aggregation depends upon secretion of other platelet agonists capable of coupling to G_i pathways. In the absence of G_i signaling by other agonists, U46619 cannot cause inhibition of adenylyl cyclase or platelet aggregation. We provide evidence for the involvement of the $P2T_{AC}$ and α_{2A} -adrenergic receptors as well as other G_i -coupled receptors in U46619-induced platelet aggregation.

EXPERIMENTAL PROCEDURES

Materials—Adenosine-3'-phosphate-5'-phosphate (ASP5P), epinephrine, apyrase (type V), ADP, fibrinogen (type I), and bovine serum albumin (fraction V) were from Sigma. The acetoxymethyl ester of Fura PE-3 was from Teflabs (Austin, TX). The stable thromboxane/prostaglandin endoperoxide analogue 9,11-dideoxy-9,11-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) and Ro 31-8220 (bisindolylmaleimide IX) were from Biomol (Plymouth Meeting, PA). Imipramine was purchased from ICN (Costa Mesa, CA). Bovine thrombin was from Parke-Davis. SC-57101 was a gift from Searle and Co. AR-C66096 (previously known as ARL 66096) was a gift from Astra Research Laboratories-Charnwood, Loughborough, UK (formerly Fisons). Yohimbine and cyproheptadine were purchased from Research Biologicals International (Natick, MA). All other chemicals were reagent grade, and deionized water was used throughout.

Isolation of Platelets—Human blood was collected from a pool of informed healthy volunteers all of whom are students or staff at Temple University School of Medicine. The donated blood was collected into a one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 ml of deionized H_2O). Platelet-rich plasma (PRP) was isolated by centrifugation of citrated blood at $180 \times g$ for 15 min at room temperature. PRP was incubated with 1 mM acetylsalicylic acid (aspirin treated) for 1 h at $37^\circ C$ followed by centrifugation at $1000 \times g$ for 10 min at room temperature. The platelet pellet was resuspended in HEPES-buffered Tyrode's solution (138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.0 mM NaH_2PO_4 , 5 mM glucose, 10 mM HEPES, adjusted to pH 7.4) supplemented with 0.2% bovine serum albumin, and 0.05 units/ml apyrase. The platelet count was adjusted to 2×10^8 cells/ml. All experiments were repeated at least three times using platelets from different donors.

Analysis of Platelet Aggregation and Shape Change—Agonist-induced platelet aggregation was determined by measuring the transmission of light through a 0.5-ml sample of aspirin-treated washed platelets (2×10^6 cells/ml) with stirring (900 rpm) in a lumi-aggregometer at $37^\circ C$ (Chrono-Log, Havertown, PA). The recorder output speed was set to 0.2 mm/s. The base line was set using 0.5 ml of HEPES-buffered Tyrode's solution as a blank. Aggregation of washed platelets required the addition of fibrinogen (1 mg/ml) prior to the addition of an agonist. Platelet shape change was observed by the addition of 1 μM SC-57101 before agonist stimulation. SC-57101 is a known inhibitor of platelet aggregation through blocking fibrinogen binding to its receptor (25). All experiments were performed in the presence of 2 mM $CaCl_2$ which was added first before either fibrinogen or SC-57101. All experiments were repeated at least three times using platelets from different donors.

Measurement of Platelet Secretion—Platelet secretion was determined by measuring the release of [3H]5-HT and expressed as the percentage of the total [3H]5-HT content. The activation of labeled [3H]5-HT platelets was performed in the lumi-aggregometer at $37^\circ C$ with stirring (900 rpm) and was stopped after 2 min with the addition of formaldehyde/EDTA according to the method of Costa and Murphy (26). Imipramine was added to the HEPES-buffered Tyrode's solution at a final concentration of 1 μM in order to prevent re-uptake of secreted [3H]5-HT. Samples were collected and centrifuged at $5000 \times g$ for 1 min, and the radioactivity of the supernatant was measured using an LKB (Amersham Pharmacia Biotech) liquid scintillation counter.

Measurement of Cytoplasmic Concentrations of Ionized Ca^{2+} —Plate-

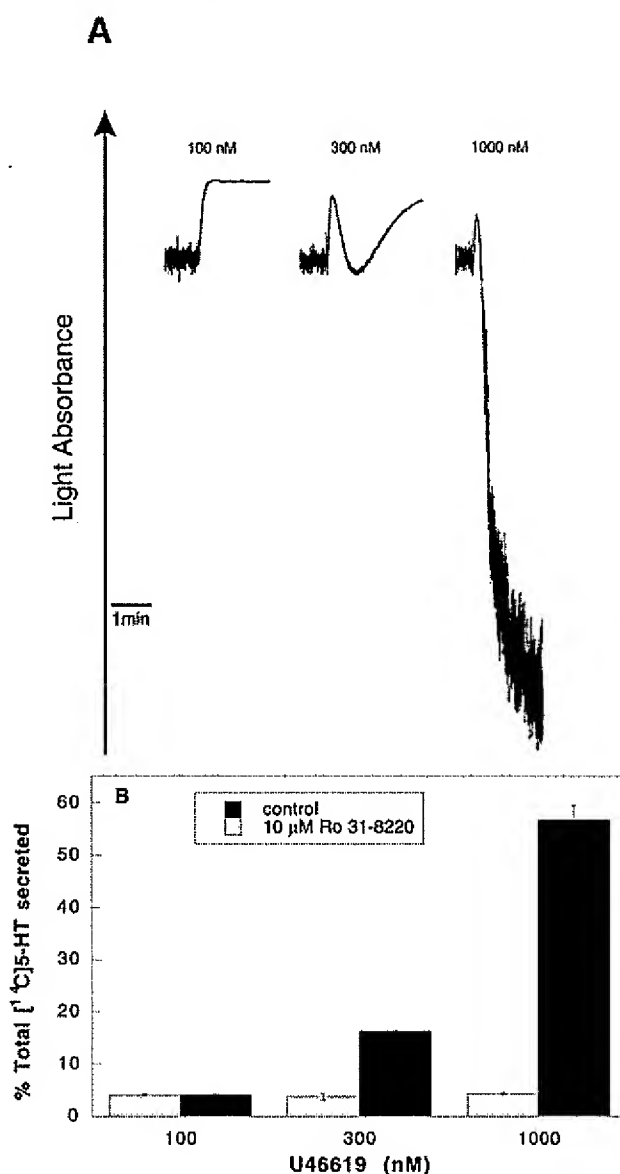


FIG. 1. Effect of varying concentrations of the TxA_2 mimetic, U46619, on platelet shape change and aggregation (A) and secretion of [3H]5-HT (B). Aspirin-treated human platelets were loaded with [3H]5-HT, washed, and resuspended in HEPES-buffered Tyrode's solution including 1 μM imipramine to prevent re-uptake of secreted 5-HT. A, platelet aggregation was measured in the presence of extracellular fibrinogen (1 mg/ml) and 2 mM $CaCl_2$ as described under "Experimental Procedures." Aggregation was performed in a cuvette maintained at $37^\circ C$ with stirring. The ordinate represents the observed changes in light absorbance (optical density) due to light scattering by the platelets. These tracings are representative of results observed on three separate occasions from three different donors. B, Ro 31-8220 or dimethyl sulfoxide (control) was added to a 0.5-ml volume of platelets and incubated for 5 min at $37^\circ C$ with stirring before the addition of U46619. Each data point is the mean \pm S.E. of three measurements. The experiment was repeated three times using platelets from different donors.

let-rich plasma was incubated at $37^\circ C$ with 3 μM Fura PE-3 acetoxymethyl ester and 1 mM acetylsalicylic acid for 45 min followed by 15 min at room temperature. The platelet-rich plasma was centrifuged at $1000 \times g$ for 10 min at room temperature. The platelet pellet was resuspended in HEPES-buffered Tyrode's solution supplemented with 0.2% bovine serum albumin, and 0.05 units/ml apyrase. The platelet count was adjusted to 2×10^8 cells/ml. Aliquots (1.0 ml) of the platelet suspension were stirred (900 rpm) in a water-jacketed cuvette maintained at $37^\circ C$ during activation. Fluorescence was constantly measured using a Perkin-Elmer LS-5 spectrofluorimeter with settings of 340

(excitation) and 510 nm (emission). Fura PE-3 fluorescence signals were calibrated as described previously (27). F_{\min} was determined by the addition of 2 mM EGTA, 20 mM Tris base, and 40 μ M digitonin. F_{\max} was determined by addition of a saturating concentration of CaCl_2 to the lysed cells. All experiments were performed in the presence of 2 mM CaCl_2 and repeated at least three times using platelets from different donors. Calibration curves for experiments that included Ro 31-8220 were performed in the presence of Ro 31-8220 due to its slight quenching of the fluorescent signal.

Measurement of cAMP—PRP was incubated with 2 $\mu\text{Ci/ml}$ [^3H]ade-

nine and aspirin (1 mM) for 1 h at 37 °C. Platelets were isolated from PRP by centrifugation as described above and resuspended in HEPES-buffered Tyrode's solution. Reactions were stopped with 1 M HCl, and 4,000 dpm of [^{14}C]cAMP was added as the recovery standard. The level of cAMP was determined as described previously (28) and measured as a fraction of total [^3H]adenine nucleotides. Results are normalized to the level of forskolin (20 μM)-stimulated cAMP and expressed as a percentage.

RESULTS

Effect of Ro 31-8220, a Protein Kinase C Inhibitor, on U46619-induced Platelet Responses—Platelets respond to increasing concentrations of ADP by first undergoing shape change and then, at a higher concentrations, aggregation (29). This is because ADP-induced platelet shape change results from activation of the high affinity P2Y_1 receptor (19), and higher concentrations of ADP are needed for co-stimulation of both the high affinity P2Y_1 receptor and a low affinity P2T_{AC} receptor to induce aggregation (19). In order to determine if concomitant higher affinity G_q -coupled signaling and lower affinity G_i -coupled signaling also occurs in response to U46619 and to determine whether aggregation requires lower concentration of U46619 than secretion, we exposed platelets to different concentrations of the agonist. Similar to the response observed for ADP, the platelets first responded to lower concentrations of U46619 (100 nM) by changing shape. Aggregation occurred at significantly higher concentrations (300 nM) (Fig. 1A). Secretion did not occur at concentrations of U46619 below 300 nM (Fig. 1B); furthermore, the onset of aggregation appears to correlate with the initiation of secretion. PKC has been shown to play an important role in the induction of platelet secretion, and secretion can be blocked using the cell-permeable inhibitor of PKC, Ro 31-8220 (30–32). We investigated the role of secretion in platelet aggregation in response to ADP, thrombin, and U46619. Secretion in response to U46619 is totally abolished by 10 μM Ro 31-8220 (Fig. 1B). In the presence of Ro 31-8220, U46619 caused shape change but did not induce aggregation (Fig. 2). Platelet aggregation induced by thrombin

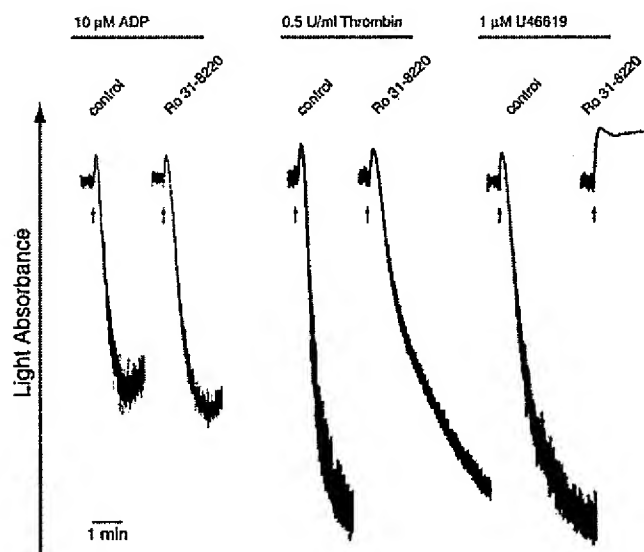


FIG. 2. Effect of 10 μM Ro 31-8220 on agonist-induced platelet aggregation. Platelet aggregation was measured as described. Aspirin-treated platelets were previously treated with either vehicle (dimethyl sulfoxide) and are labeled control or with 10 μM Ro 31-8220 as indicated. The arrow indicates the addition of agonist as indicated into a cuvette maintained at 37 °C with stirring. 2 mM extracellular CaCl_2 was previously added to the cuvette. The tracings are representative of three experiments.

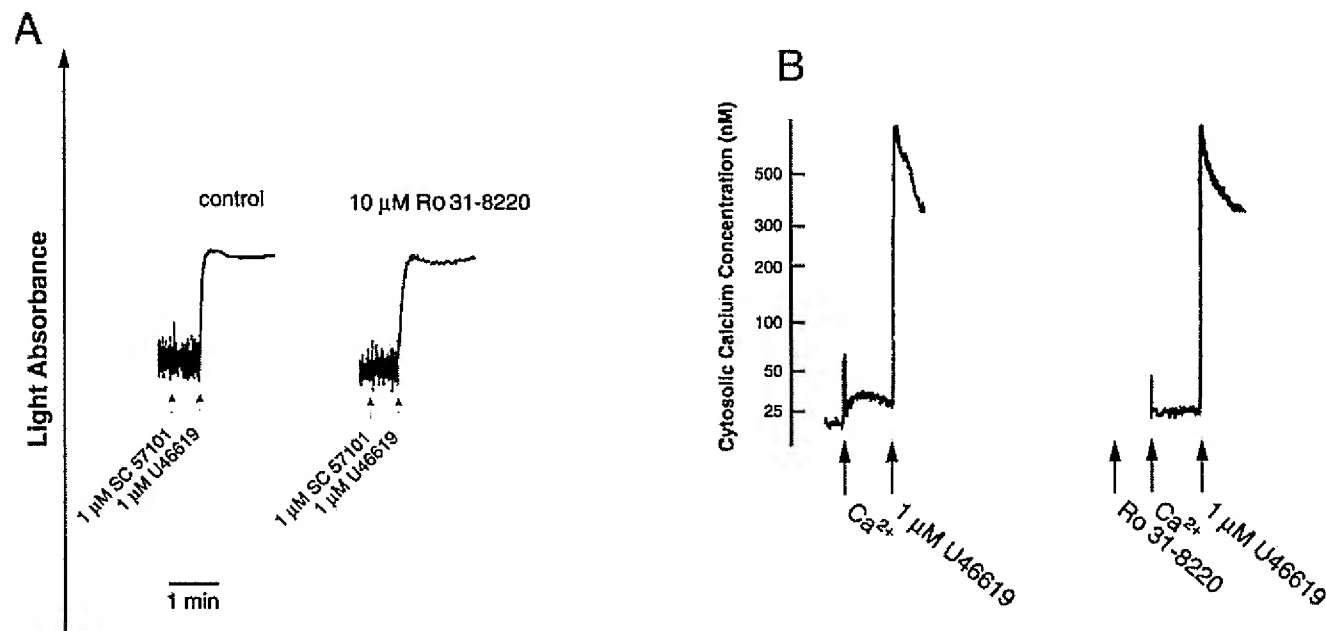


FIG. 3. The effect of Ro 31-8220 on U46619-induced platelet shape change (A) and Ca^{2+} mobilization (B). A, platelet shape change was induced by 1 μM U46619 (addition of agonist indicated by the arrow) in aspirin-treated platelets that were previously treated with either vehicle (dimethyl sulfoxide, labeled control) or with 10 μM Ro 31-8220 as indicated. 2 mM extracellular CaCl_2 was previously added to the cuvette. U46619-induced platelet shape change was analyzed in the presence of 1 μM SC-57101 (addition indicated by arrow). The tracings are representative of three experiments. B, aspirin-treated platelets labeled with Fura PE3 were treated with either vehicle (dimethyl sulfoxide) or with 10 μM Ro 31-8220 (5 min) and then stimulated with 1 μM U46619 in a cuvette maintained at 37 °C with stirring (900 rpm). Labeled arrows indicate addition of Ro 31-8220, 2 mM CaCl_2 , and U46619. The tracings are representative of three experiments using platelets from different donors.

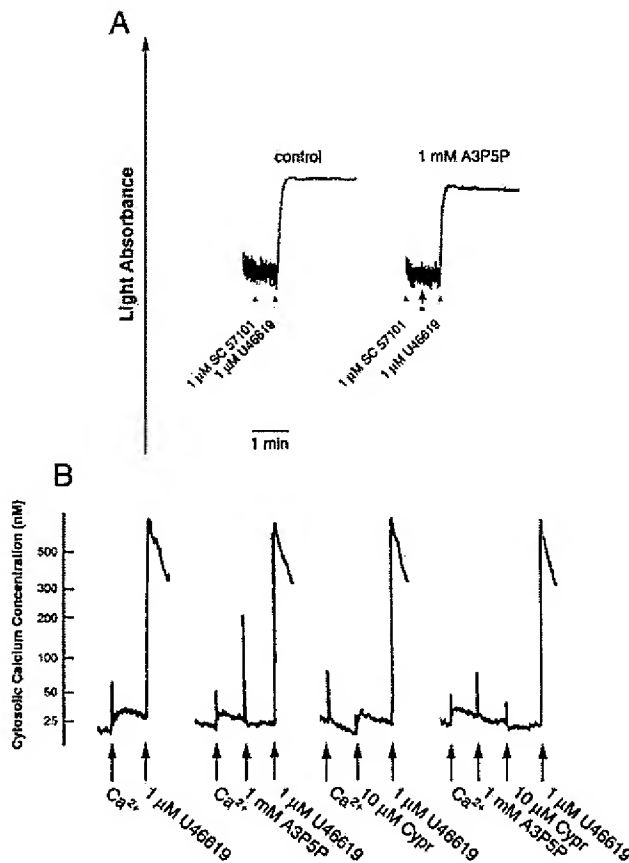


FIG. 4. Effect of receptor-selective antagonists on U46619-induced platelet shape change (A) and intracellular calcium mobilization (B). A, platelet shape change was induced by 1 μ M U46619 either in the presence or absence of 1 mM A3P5P (addition indicated by arrow with asterisk) under conditions previously described. U46619-induced platelet shape change was analyzed in the presence of 1 μ M SC-57101. Addition of reagents is indicated by arrows. The tracings are representative of three experiments using platelets from different donors. B, aspirin-treated platelets labeled with Fura PE3 were treated with either 1 mM A3P5P or 10 μ M cyproheptadine (abbreviated as Cypr) as indicated and then stimulated with 1 μ M U46619 as described previously. Arrows labeled Ca^{2+} indicate the addition of 2 mM extracellular CaCl_2 . The tracings are representative of three experiments using platelets from different donors.

was slightly slowed down indicating the participation of secreted agonists, whereas aggregation in response to ADP was unaffected (Fig. 2).

Effect of Ro 31-8220 on U46619-induced G_q -coupled Platelet Responses—ADP-mediated G_q -coupled signaling has been shown to be required for both platelet shape change and aggregation (19, 28). Stimulation of the TP receptor with 30–100 nM U46619 leads to platelet shape change resembling selective stimulation of the G_q -coupled P2Y₁ receptor. In order to assess the possible effects of secretion on G_q -mediated signaling, both platelet shape change and intracellular Ca^{2+} mobilization were measured in the presence and absence of Ro 31-8220, a protein kinase C (PKC) inhibitor. Platelet shape change in response to U46619 was not affected by Ro 31-8220 (Fig. 3A) indicating that these signaling pathways are not dependent upon either secretion or PKC activity. Furthermore, the U46619-induced increase in cytosolic Ca^{2+} was unaffected by the presence of Ro 31-8220 (Fig. 3B) indicating that G_q -coupled signaling initiated by TP receptor stimulation is independent of released granule contents.

Effect of Receptor-selective Antagonists on U46619-induced G_q -coupled Platelet Responses—Platelet secretion releases

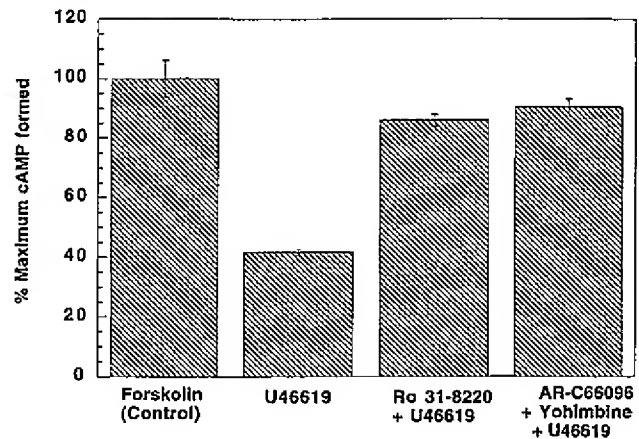


FIG. 5. Effect of Ro 31-8220 or receptor-selective antagonists on U46619-induced inhibition of platelet adenylyl cyclase. Data are collected as the fractions of total nucleotides that are [^3H]cAMP. The data are normalized to the level of forskolin-stimulated cAMP (taken as 100%) or to the level of forskolin-stimulated cAMP in the presence of 10 μ M Ro 31-8220. Ro 31-8220 or dimethyl sulfoxide (control) was added to a 0.5-ml volume of aspirin-treated platelets and incubated for 5 min at 37 $^{\circ}\text{C}$ with stirring (900 rpm) before the addition of either 20 μ M forskolin alone or 20 μ M forskolin with 1 μ M U46619. 1 μ M AR-C66096 and 10 μ M yohimbine were added 1 min before the addition of 1 μ M U46619.

ADP and serotonin at the site of injury in order to activate and recruit more platelets into the forming primary hemostatic plug (2). By using receptor-selective antagonists, we investigated the contribution of these agonists to U46619-induced G_q -coupled responses. The compound A3P5P is an antagonist of the G_q -coupled P2Y₁ receptor (33). Cyproheptadine is an antagonist at the 5-HT_{2A} receptor (34–37). Aggregation was not affected by the presence of either compound (data not shown). U46619-induced platelet shape change was not affected by the presence of A3P5P (Fig. 4A) or cyproheptadine (not shown) indicating the lack of any contribution by the P2Y₁ or serotonin receptors to this event. The possible contribution of both the P2Y₁ and 5-HT_{2A} receptors in the mobilization of intracellular Ca^{2+} was investigated. Intracellular Ca^{2+} mobilization in response to U46619 was not affected by A3P5P and/or cyproheptadine (Fig. 4B).

Effect of Ro 31-8220 or Receptor-selective Antagonists on U46619-induced Inhibition of Platelet Adenylyl Cyclase—Previous studies have shown that U46619 causes a decrease in the intracellular concentration of cAMP in platelets (38, 39). In order to determine whether TP receptors can couple to G_i -signaling pathways, we utilized two approaches. The first was to block secretion using Ro 31-8220. In the absence of Ro 31-8220, U46619 inhibited forskolin-stimulated adenylyl cyclase (Fig. 5). In the presence of Ro 31-8220, U46619 failed to inhibit adenylyl cyclase. The second approach utilized receptor-selective antagonists to the P2T_{AC} and α_{2A} -adrenergic receptors. AR-C66096 is an antagonist at the G_i -coupled P2T_{AC} receptor (28), and yohimbine is an antagonist at the G_i -coupled α_{2A} -adrenergic receptor (40, 41). Platelet dense granules contain both ADP and epinephrine which cause the inhibition of cAMP following activation at their respective receptors (2). The level of cAMP was measured following stimulation of platelets in the absence and presence of the antagonists AR-C66096 and yohimbine. These antagonists effectively prevented the contribution of G_i -coupled signaling by either the P2T_{AC} or the α_{2A} -adrenergic receptor, respectively. As shown in Fig. 5, U46619-induced adenylyl cyclase inhibition was also blocked by these receptor antagonists, suggesting that U46619-induced G_i stimulation depends on secreted ADP and epinephrine.

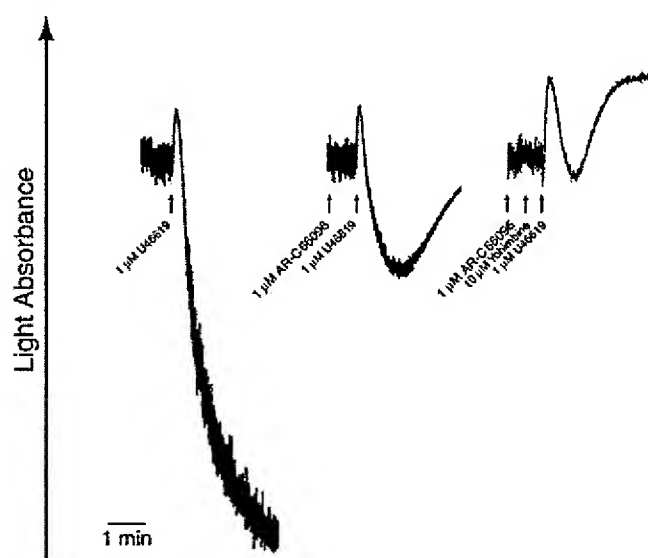


FIG. 6. Effect of the $P2T_{AC}$ antagonist, AR-C66096, and the α_2 -adrenergic antagonist, yohimbine, on U46619-induced platelet aggregation. Platelet aggregation was measured as described previously. The arrows indicate the addition of $1 \mu\text{M}$ AR-C66096 or $10 \mu\text{M}$ yohimbine into a cuvette maintained at 37°C with stirring. 2 mM extracellular CaCl_2 was previously added to the cuvette. The tracings are representative of three experiments.

Effect of Receptor-selective Antagonists on U46619-induced Platelet Aggregation—We (18) and others (20, 21) have provided evidence that concomitant signaling through both G_i -coupled and G_q -coupled receptors is required for platelet aggregation. Since the TP receptor does not couple to G_i , independently of secreted ADP and epinephrine (Fig. 5), we utilized receptor-selective antagonists to elucidate the role of these G_i -coupled receptors in U46619-induced platelet aggregation. AR-C66096 dramatically inhibited ADP-induced platelet aggregation (18, 28). The rate and extent of U46619-induced aggregation were diminished in the presence of AR-C66096 (Fig. 6). In the presence of AR-C66096, yohimbine further inhibited U46619-induced platelet aggregation (Fig. 6). However, yohimbine alone was without any significant effect (not shown). These results indicated that the $P2T_{AC}$ receptor is essential for U46619-induced platelet aggregation.

Restoration of U46619-induced Aggregation Blocked by Ro 31-8220—In order to verify that signaling through a G_i -coupled receptor only occurs following U46619-induced secretion, we investigated the effects of selective activation of G_i -coupled receptor stimulation in the presence of Ro 31-8220. Control experiments were performed to ensure that platelets respond normally to ADP and thrombin in the presence of Ro 31-8220 or vehicle (not shown). The $P2T_{AC}$ receptor was selectively activated by ADP in the presence of A3P5P. As shown in Fig. 7, selective activation of the $P2T_{AC}$ receptor reversed the effects of secretion blockade on U46619-induced aggregation. AR-C66096 blocked this reversal, providing further evidence that ADP is selectively activating the G_i -coupled $P2T_{AC}$ receptor (Fig. 7). Epinephrine also reversed the inhibitory effects of Ro 31-8220 on U46619-induced aggregation. Addition of ADP and epinephrine together potentiated this reversal. Thus platelet aggregation in response to U46619 is mediated by concomitant signaling through the G_q -coupled TP receptor and the G_i -coupled $P2T_{AC}$ and α_{2A} receptors.

DISCUSSION

The molecular mechanisms leading to aggregation following platelet exposure to thromboxane A_2 have yet to be clearly

elucidated. Four explanations for the stimulatory action caused by U46619 or other thromboxane A_2 mimetics are possible. First, U46619 may activate G_q and G_i through the $TP\alpha$ and $TP\beta$ receptors, respectively. Second, it is conceivable that U46619 only activates the G_q pathway and that secreted ADP activates the G_i pathway. Although unlikely, a third explanation is that U46619 activates G_i or G_o through $TP\beta$ leading to the activation of phospholipase C and the inhibition of cyclase. Following secretion, released ADP would activate the G_q pathway. Finally, U46619 may activate an unidentified G protein-coupled pathway that results in secretion of ADP which activates both G_q and G_i through the $P2Y_1$ and the $P2T_{AC}$ receptors, respectively. We used three complementary approaches to identify the molecular mechanisms of U46619-induced platelet activation as follows: 1) determination of the minimum concentration required for platelet aggregation and secretion by U46619, 2) blockade of secretion, and 3) receptor subtype-selective antagonists in order to eliminate the positive feedback from granule contents. Here we report that although thromboxane A_2 causes intracellular calcium mobilization and shape change independently, thromboxane A_2 -induced inhibition of adenylyl cyclase and platelet aggregation depend exclusively on ADP and other released granule contents.

Evidence exists for a dissociation of platelet activation responses following stimulation of the TP receptor. First, the EC_{50} values of the TP receptor agonists, U46619 (42) and STA_2 (43), for an increase in cytosolic Ca^{2+} and platelet shape change are lower than the EC_{50} values for secretion and aggregation. Our data indicate that platelet aggregation correlates with the occurrence of secretion. We observed that platelet shape change occurs at lower concentrations of U46619 and that aggregation occurs at higher concentrations (Fig. 1A). Furthermore, the same concentration of U46619 that leads to the initiation of aggregation also initiates secretion (Fig. 1B). However, from this evidence it is not clear if platelet aggregation results in part from P2 receptor stimulation.

Substantial evidence exists that PKC activation is required for platelet secretion (31). In platelets activated by U46619 in the presence of Ro 31-8220, it was reported that P47 phosphorylation, fibrinogen binding, and serotonin release were all inhibited (32). In agreement with previous studies, our results show that Ro 31-8220 prevented U46619-induced platelet aggregation (Fig. 2). We observed that Ro 31-8220 inhibited U46619-induced secretion in platelets loaded with [^{14}C]serotonin in the presence of 2 mM Ca^{2+} (Fig. 1B) and that Ro 31-8220 did not inhibit the increase in cytosolic Ca^{2+} induced by U46619 (Fig. 3B).

Ro 31-8220 failed to inhibit ADP- or thrombin-induced platelet aggregation (Fig. 3) suggesting that the Ro 31-8220 inhibitable PKC isoforms do not directly contribute to fibrinogen receptor activation. Ro 31-8220 has been shown to block PKC isoforms α , β , γ , and ϵ (44). Hence these PKC isoforms do not contribute to the inside-out signaling leading to fibrinogen receptor activation by either ADP or thrombin.

Considering that secretion and aggregation both occur at the same concentration of U46619 (Fig. 1) and that blocking secretion prevents aggregation (Fig. 2), it is reasonable to suggest that thromboxane A_2 -induced aggregation is dependent upon secretion. The role of ADP in thromboxane A_2 -induced platelet aggregation has been investigated using enzymes that deplete released ADP. This work suggested that the aggregation response is mediated by the secretion of platelet ADP (45–49). It was concluded that U46619-induced platelet aggregation depends on the release of stored ADP. The use of apyrase could have enhanced the generation of adenosine from AMP. Adenosine binds to the G_s -coupled A_2 receptor resulting in an in-

FIG. 7. Restoration of Ro 31-8220 blocked aggregation by selective stimulation of either the $P2T_{AC}$ receptor or the α_2 -adrenergic receptor. Platelet aggregation was measured as described. Aspirated platelets were previously treated with either vehicle (dimethyl sulfoxide) and are labeled control or with 10 μ M Ro 31-8220 as indicated. Antagonists added before U46619 are indicated above tracings. The arrows indicate the addition of 1 μ M U46619, 10 μ M ADP, or 10 μ M epinephrine (indicated as *EPI*). All additions were made into a cuvette maintained at 37 °C with stirring. 2 mM extracellular $CaCl_2$ was previously added to the cuvette. Not shown are the control responses of platelets to ADP and thrombin in the presence of Ro 31-8220 or vehicle. The tracings are representative of three experiments.

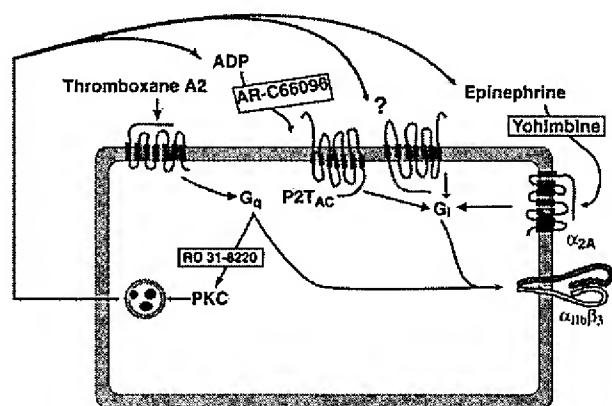
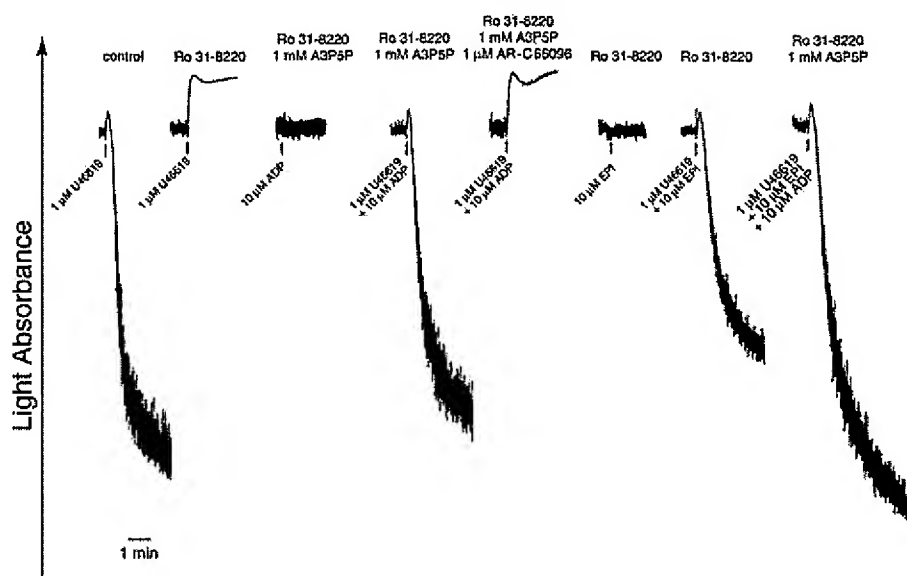


FIG. 8. Model depicting the molecular events of thromboxane A_2 -induced platelet activation. Solid arrows depict stimulatory pathways. Receptor-selective antagonists are indicated in boxed type. U46619 acts at the G_q -coupled TP receptor to cause secretion of granule contents. Activation of G_i -coupled receptors is dependent upon secretion. Concomitant stimulation of G_q -coupled and G_i -coupled receptors leads to platelet aggregation.

crease in the intracellular concentration of cAMP and inhibits platelet activation (50, 51). Moreover, these studies did not clearly determine how ADP and the other components of the dense and α -granules contribute to TxA_2 -induced platelet aggregation. The use of creatine phosphate/creatine phosphokinase converts ADP to ATP, an antagonist at the platelet ADP receptors (2). ATP can also potentially stimulate adenylyl cyclase activity resulting in inhibition of platelet activation (52, 53). Our experiments make use of the receptor subtype-selective antagonists AR-C66096 and yohimbine, which block stimulation of G_i signaling.

Evidence exists to support the presence of the $TP\alpha$ and $TP\beta$ receptor subtypes in platelets (8, 17); these isoforms, when expressed in Chinese hamster ovary cells, have been shown to couple to G_s and G_i pathways, respectively. However, in the presence of Ro 31-8220, high concentrations of U46619 did not alter the level of cAMP, indicating that TP receptor subtypes do not couple to adenylyl cyclase in platelets. Our observation also is supported by two studies. By using platelet membranes, U46619 was found to have no effect upon levels of cAMP (54). Furthermore, Klages *et al.* (55) have shown that U46619 does not stimulate G_i proteins in mouse platelets. G protein coupling

may be affected by levels of heterologous receptor expression; furthermore, high levels of receptor expression can lead to promiscuous coupling to multiple G proteins.

U46619-induced aggregation requires concomitant stimulation of both a G_q -coupled receptor and a G_i -coupled receptor. Granule contents appear to mediate the stimulation of G_i -coupled signaling as is evident by the lack of cyclase inhibition when U46619-induced platelet secretion is prevented (Fig. 5). The fact that signaling through the G_q -coupled TP receptor is unaffected under such conditions is apparent by both the robust shape change response (Fig. 3A) and the normal level of cytosolic Ca^{2+} mobilization (Fig. 3B).

An alternative explanation for the effect of Ro 31-8220 on U46619-induced platelet aggregation is that U46619 causes platelet aggregation involving activation of a PKC isoform through a mechanism different from that of ADP. Hence, Ro 31-8220 would inhibit U46619-induced aggregation by inhibiting this PKC isoform in addition to blocking secretion. This possibility was ruled out using receptor-selective antagonists.

Through the use of receptor-selective antagonists, we were able to identify clearly the contribution of receptors mediating aggregation following U46619-induced secretion. Antagonists at G_q -coupled receptors such as cyproheptadine and A3P5P had no effect on aggregation, shape change, or the increase in cytosolic Ca^{2+} concentration. In contrast, both of the G_i -coupled $P2T_{AC}$ and α_{2A} -adrenergic receptors were found to mediate aggregation and inhibition of adenylyl cyclase, following U46619-induced secretion (Fig. 6). The compound AR-C66096 had the greatest inhibitory effect indicating the large contribution to G_i -coupled signaling by the $P2T_{AC}$ receptor. In the absence of AR-C66096, yohimbine failed to affect U46619-induced aggregation, indicating that G_i stimulation could be compensated by $P2T_{AC}$ receptor stimulation. The amount of epinephrine found in platelets is extremely small (1.1–3.8 pmol/ 1×10^8 platelets) (56); however, the initial concentration of this secreted amount in the microenvironment of the platelet could be much greater. As observed, the secreted epinephrine makes a significant contribution as revealed by the inhibition of aggregation by yohimbine only in the absence of $P2T_{AC}$ receptor stimulation (Fig. 6). This suggests that secretion of the G_i -coupled receptor stimulating agonists (ADP and epinephrine) are required for full aggregation following activation of G_q -coupled signaling by thromboxane A_2 . When U46619-induced secretion was blocked by Ro 31-8220 aggregation was pre-

vented. Under these conditions the selective activation of either the G_i -coupled $P2T_{AC}$ receptor or the α_{2A} -adrenergic receptor restored aggregation (Fig. 7).

Further evidence for the important role of the $P2T_{AC}$ receptor in mediating the platelet response to TxA_2 is provided by reports of patients with congenital ADP receptor defects (57–59). In these cases the shape change and cytosolic Ca^{2+} mobilization responses to ADP are present indicating function of the $P2Y_1$ receptor, whereas ADP-induced aggregation and inhibition of adenylyl cyclase are absent. Such findings suggest that the defect involves the $P2T_{AC}$ receptor. The lack of signaling due to a defective $P2T_{AC}$ receptor affects the response of these platelets to thromboxane A_2 mimetics. In both cases, U46619-induced activation of the integrin $\alpha_{IIb}\beta_3$ was inhibited (58, 59). Inhibition of adenylyl cyclase by epinephrine in platelets from both patients was normal, suggesting that the residual fibrinogen receptor activation could be due to activation of α_{2A} -adrenergic receptors by secreted epinephrine. On the other hand, we predict that in the case of a hypothetical $P2Y_1$ receptor defect, platelet aggregation in response to U46619 would appear normal as G_i stimulation, although the $P2T_{AC}$ receptor and the α_2 -adrenergic receptor would be intact.

Even in the presence of both AR-C66096 and yohimbine we still observed some residual aggregation (Fig. 6). We propose that this residual aggregation results from G_i signaling by other components of the granules. This prediction is supported by the fact that secretion blockade completely eliminates U46619-induced platelet aggregation. Based on previous and recent reports describing the mechanism of action by thrombospondin, a major constituent of the α granules, in platelet activation and aggregation (60–62), we suggest that it too may be mediating TxA_2 mimetic-induced aggregation. A recent study has demonstrated that thrombospondin can stimulate the G_i -signaling pathways (60).

In conclusion, as outlined in Fig. 8, our results show that U46619 causes platelet shape change and intracellular Ca^{2+} mobilization independently of secreted granule contents. However, U46619-induced platelet aggregation depends exclusively on G_i stimulation by ADP and other released granule contents. The $P2T_{AC}$ receptor appears to be the predominant stimulator of the G_i pathway. These results further support the hypothesis that platelet fibrinogen receptor activation requires concomitant signaling from the G_q - and G_i -signaling pathways.

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Exhibit 24

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9

Specific Thrombin Inhibitors

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Thrombin represents the culmination of the coagulation cascade as it converts fibrinogen to clottable fibrin by releasing fibrinopeptides A and B. Thrombin itself is responsible for its own nonlinear generation caused by positive feedback activation, whereby thrombin enhances neoformation of thrombin (Fig. 9-1). In addition, thrombin is a pivotal molecule for numerous other functions. By binding to its

receptor and subsequent cleaving, thrombin is the most potent known platelet activator. The action of thrombin on platelets results in the release of platelet factor V exteriorization and the transbilayer movement of its inner membrane surface (flip-flop reaction). Thrombin activates three of the four cofactor or helper proteins (factors V and VIII, thrombomodulin, but not tissue factor). Thrombin further-

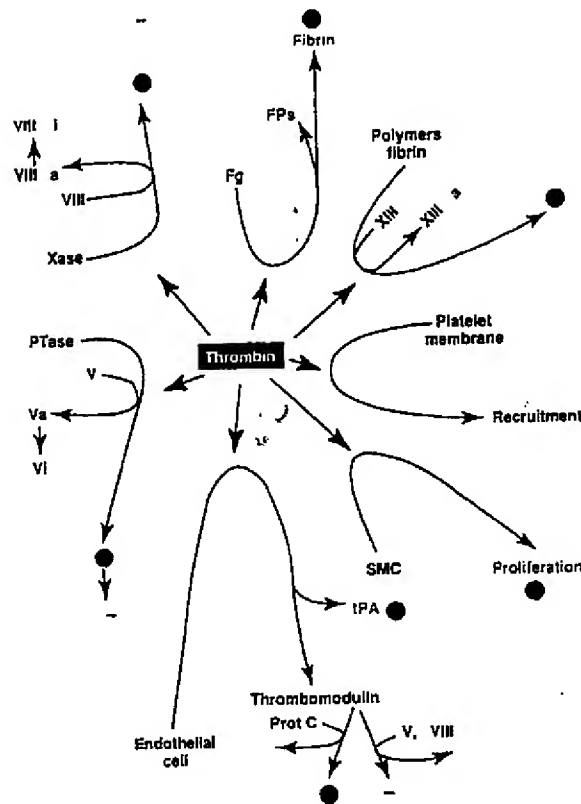


FIG. 9-1. Role of thrombin in the pathogenesis of arterial thrombosis. Positive signs (+) indicate actions stimulated by thrombin, whereas negative signs (-) indicate reactions inhibited by it. In addition to its effects on the activation of coagulation factors and fibrin formation and stabilization, thrombin activates platelets, induces proliferation of smooth muscle cells (SMC), and contributes to activation of the spontaneous anticoagulant pathway of normal endothelium. Fps, fibrinopeptide; Fg, fibrinogen. (From Badimon and Badimon, ref. 79, with permission.)

more activates factor XIII, which increases the strength and renders the fibrin more resistant to thrombolysis.

Thrombin also may prevent coagulation by a negative feedback mechanism. When thrombin binds thrombomodulin on endothelial cells, it cleaves and activates protein C, a natural anticoagulant, which in turn inactivates factors Va and VIIa. Protein S specifically accelerates the degradation of factor V catalyzed by activated protein C.

Thrombin in association with intact endothelium induces the production and release from the vascular endothelial cells of two

highly potent local antiaggregatory vasodilators: prostacyclin and nitric oxide (endothelium-derived relaxing factor). These molecules are thought to provide significant antithrombotic protection for microcirculatory beds adjacent to sites of thrombus formation.

In vivo thrombin receptor expression also demonstrated by macrophages and vascular smooth muscle cells in atherosclerotic lesions and in endarterectomy specimens. Expression of selectins at the endothelial cell surface is stimulated by thrombin and plays a role in the incursion of monocytes and neutrophils into an injured vessel wall. In addition, thrombin

has a direct chemotactic effect on monocytes and has apparent mitogenic effects on lymphocytes and vascular smooth muscle cells.

Considering the pivotal role of thrombin in the coagulation system, substantial research is focused on specific inhibitors of thrombin. What they all have in common is that unfortunately no specific antidote is presently available.

DESULFATOHIRUDIN

Biochemistry and Experimental In Vivo Studies

Several natural hirudin variants (iso-inhibitors), with different N-terminal amino acids, are produced by the salivary gland of the European leech, *Hirudo medicinalis*, and are the prototype of direct antithrombins (1,2). Natural desirudin is a 65-residue polypeptide with three intramolecular disulfide bonds and a

sulfated tyrosine residue in position 63. It has a molecular weight of approximately 7 kDa and binds thrombin with extraordinary tightness (dissociation constant $[K_D]$ 2×10^{-15} mol/L) and specificity (3,4), which is the result of 212 close ($< 4 \text{ \AA}$) contacts between inhibitor and enzyme (4). Hirudin binds thrombin with 1:1 stoichiometry. The highly negatively charged C-terminus of hirudin interacts with the anion-binding exosite of thrombin, whereas its apolar domain (residue 1–48), stabilized by the three disulfide bridges, interacts with a region adjacent to the amidolytic center of thrombin (Fig. 9-2). Multiple nonpolar contacts contribute to the exceptional affinity of the thrombin–hirudin complex (2). Recombinant desirudin is produced in *Escherichia coli* and yeast and lacks the sulfate residue on tyrosine-63, with the consequence that its affinity toward thrombin is decreased by one order of magnitude (to K_D 2×10^{-13} mol/L) relative to the natural sulfated form (Table 9-1) (5). Nonetheless, with the exception of natural hirudin, desirudin has by far the highest affinity toward thrombin of all known antithrombins.

The efficacy of hirudin was believed until recently to be based on direct thrombin inhibition and especially on the interruption of thrombin generation by inhibiting the positive feedback that thrombin exerts through multiple mechanisms on its own generation. When assembled on lipid (e.g., platelet membranes), the prothrombinase complex generates thrombin 280,000 times faster than when each component is present in solution. Self-amplifica-

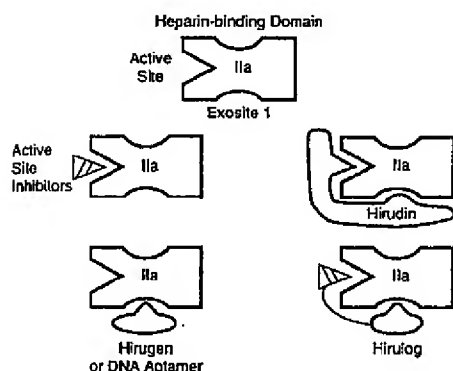


FIG. 9-2. Interaction of direct thrombin inhibitors with thrombin. In addition to the active site of thrombin (IIa), there are two distinct positively charged domains located at opposite poles of the enzyme. The first, known as anion-binding exosite 1, serves as the substrate recognition site, whereas the second, which has been designated exosite 2, contains the heparin-binding domain. Active-site inhibitors (e.g., D-Phe-Pro-Arg CH₂ Cl and its derivatives) block the active site of thrombin, whereas hirugen and the thrombin-binding DNA aptamer bind to exosite 1, thereby preventing the enzyme from interacting with its substrates. In contrast, hirudin and hirulog bind both with the active site and with exosite 1. (From Weltz, ref. 80, with permission.)

TABLE 9-1. Affinity for thrombin of experimental antithrombins

Inhibitor	K_D of thrombin-inhibitor complex (mol/L)
r-Hirudin	2.0×10^{-13}
DuP 714	4.1×10^{-11}
Hirulog	2.3×10^9
PPACK ^a	3.7×10^6
Argatroban	3.9×10^6
Hirugen	1.5×10^7

^aThrombin and PPACK (D-Phe-Pro-ArgCH₂Cl) form an irreversible, covalent bond after initial, reversible association in a complex with the indicated K_D (dissociation constant).

tion of thrombin through activation of platelets and factors V, VIII, and X is thought to be critical for the generation of thrombin in concentrations necessary for effective hemostasis and thrombosis (6,7). Heparin (8) and the specific antithrombins (7) are thought to block thrombin generation through interruption of this positive feedback. However, doubt has been cast on this latter mechanism by the demonstration that thrombin generation *in vivo* proceeds relentlessly in the presence of desirudin and other antithrombins in animals (9) and in humans with stable and unstable coronary artery disease (10–12).

The effects of natural hirudin on the platelet-rich thrombus have been studied for over 60 years (13). Like all direct antithrombins, hirudin inhibits thrombin without the need for additional cofactors. The notion that thrombin may play a pivotal role in the formation of platelet thrombi was suggested by several and disparate early observations: the studies with crude hirudin preparations and heparin in an *ex vivo* arteriovenous shunt model of "white thrombi" by Shionoya in late 1927 (13), the observation that thrombin may initiate platelet aggregation *in vitro* at lower concentrations than those required for fibrinogen cleavage (14), and in flow-modeling experiments, where thrombin reached platelet-active concentrations significantly more rapidly compared with the thromboxane A₂ analogue U-46619 (15). More recently, in the porcine carotid injury model, specific thrombin inhibition with desirudin completely prevented formation (16), and accelerated lysis (17), of platelet-rich thrombi. In this model of platelet-rich occlusive thrombus after deep carotid artery injury, desirudin, at activated partial thromboplastin times (APTT) two to three times baseline, was found to be significantly more effective than heparin (at APTT more than fivefold baseline) in accelerating lysis by t-PA, suggesting that thrombosis after deep arterial injury was thrombin dependent. Aspirin in this model was ineffective as adjunctive to t-PA (17). In a coronary electric injury study in the dog, desirudin, at APTT values only 1.5 to 2.0 times control, was also significantly more effective than as-

pirin or heparin and prevented coronary occlusion in six of six animals (18). Desirudin also prevented thrombus deposition in *ex corporeal* Dacron-grafted arteriovenous shunts (19). Comparing the amount of desirudin required for thrombus prevention in different thrombosis models (20), it appears that the plasma concentration of desirudin required to totally prevent thrombus may be proportional to the amount of thrombin generated in these models, where an increased gradient of thrombin generation (from models of venous stasis and mild arterial injury to deep arterial injury by angioplasty in the dog) was noted (20). Like heparin (21,22), desirudin is an effective inhibitor of fibrin deposition at concentrations that do not affect platelet deposition. Thus, during disseminated intravascular coagulation in a rat model, a plasma desirudin concentration of 0.1 µg/ml was sufficient to inhibit fibrinogen deposition, whereas a concentration of 0.5 µg/ml was required to prevent deposition of platelets (23).

Pharmacodynamics and Dose-Finding Studies in Humans

In healthy young volunteers, the terminal half-life of desirudin was found to be 50 minutes, and the half-life on the APTT was 2 to 3 hours (24,25). In contrast, in older patients with stable coronary artery disease and normal renal function (serum creatinine ≤1.0 mg/dl), both plasma half-life and the half-life of the APTT prolongation was found to be 2 to 3 hours (26). In these patients, plateau/baseline APTT ratios were 1.5, 2.3, 2.7, and 2.9, respectively, with desirudin infused without initial bolus at 0.02, 0.05, 0.2, and 0.3 mg/kg/hr. Interestingly, 62%–77% of the plateau (3 to 6 hours) effect on APTT was reached within 30 minutes of start of the maintenance infusion. Plasma concentration of desirudin correlated with both the APTT/baseline ratio ($r = 0.80$) and the activated clotting time (ACT) ($r = 0.80$), although there was a considerable overlap between baseline ACT and ACT at plasma desirudin concentrations of less than 1 µg/ml. Prothrombin times (PT) were insensitive

plasma desirudin levels, with an international normalized ratio (INR) of 2.3 observed only with the highest (0.3 mg/kg/hr) dose. Thrombin times (TT) were beyond the upper range (600 seconds) in nearly all patients. Bleeding times were not significantly prolonged in this study and only mildly prolonged in another trial (26). From these studies, the APTT emerged as the best test to evaluate the anticoagulant effect of desirudin.

Immunogenicity

Desirudin has very low immunogenic potential. In repeated administration of recombinant desirudin to 263 healthy volunteers, including 12% who had a history of previous allergy and 18% with high levels of total immunoglobulin E, no signs or symptoms directly attributable to desirudin were noted, and only three of 200 volunteers exposed to a second course of desirudin showed an allergic reaction. In all but one patient with a pruritic erythema, a causative role for desirudin was excluded (27). In the same study, specific antibodies directed against desirudin were detected in only one of the 263 subjects. Thus, repeated doses of desirudin can be considered safe. Similarly, antibody formation against desirudin was not observed in an older study of volunteers (28) or in the aforementioned study in older patients with stable coronary artery disease (26).

Clinical Trials of Desirudin

Trials of Desirudin in Patients Undergoing Percutaneous Transluminal Coronary Angioplasty for Stable or Unstable Angina

In a double-blind pilot trial, van den Bos et al. randomized 113 low-risk patients with stable angina pectoris undergoing percutaneous transluminal coronary angioplasty (PTCA) to a 24-hour infusion of either desirudin (Revasc™, Novartis) or heparin (29). All patients received 250 to 500 mg aspirin for at least 4 weeks beginning on the day of PTCA. Desirudin was given as a 20-mg bolus followed by an infusion of 0.16 mg/kg/hr, whereas heparin was administered as a

10,000-IU bolus followed by 12 IU/kg/hr. The two trial drugs were adjusted to a target APTT of 85 to 120 seconds. At the end of the infusion period, an angiography was performed to assess vessel patency. Acute closure, leading to myocardial infarction and/or coronary artery bypass graft (CABG), occurred in 10.3% of patients randomized to heparin but in only 1.4% of patients randomized to desirudin. Four desirudin patients (5%) developed major bleeding at the arterial puncture site versus none in the heparin group, in which one episode of cerebral infarction occurred. Immediately and 24 hours after PTCA, TIMI grade 3 flow was present in all desirudin-treated patients and, respectively, in 92% and 91% of heparin-treated patients. None of the differences in this small pilot trial reached statistical significance, in part because of low event rates and the small numbers of patients. Desirudin provided more predictable APTT prolongations with more patients in the target APTT range. Although the prothrombin fragment 1.2 levels (an indicator of prothrombin generation) were below the upper limit of normal in both groups, they tended to be higher in the desirudin group (29).

Based on the results of this pilot trial, it was concluded that desirudin can be administered safely to patients undergoing PTCA for stable angina pectoris.

Experimental animal studies had suggested that a short-term administration of desirudin may inhibit restenosis at 1 month from angioplasty (30). This hypothesis was tested in the large Helvetica study of 1,141 patients with unstable angina in which a higher dose of desirudin than in the pilot study was used (31). Desirudin was given as a 40-mg bolus and a 24-hour infusion at 0.2 mg/kg/hr followed by either subcutaneous (s.c.) desirudin (40 mg twice daily) or placebo for three consecutive days. The heparin dose was a bolus of 10,000 IU followed by an intravenous (i.v.) infusion of 15 IU/kg/hr over 24-hour infusion and two s.c. placebo injections per day for 3 days. The incidence of early (96 hours) ischemic events was reduced significantly by desirudin versus heparin (relative risk reduction in the combined desirudin groups, 0.61; 95% confidence inter-

val [CI], 0.41 to 0.90, $p = 0.023$), which was particularly evident for those patients with angina at rest: early event rate 21.6% in the heparin versus 5.3% in patients receiving both i.v. plus s.c. desirudin (relative risk reduction 0.41; 95% CI, 0.21 to 0.78, $p = 0.006$). There was no difference in the incidence of major or minor bleeding complications. Prothrombin fragments 1.2 levels immediately after angioplasty were decreased by heparin but not by desirudin (31). Seven-month event-free survival (freedom from death, nonfatal myocardial infarction or revascularization procedure within 7 months from PTCA) was 67.3% in the heparin group, 63.5% in the hirudin i.v. group, and 68.0% in the patients treated with i.v. plus s.c. hirudin ($p = \text{NS}$). Minimal lumen diameters at 6 months' follow-up angiography were 1.54 mm, 1.46 mm, and 1.56 mm, respectively, in the three treatment groups. In conclusion, compared with heparin, desirudin in this trial in unstable angina patients treated with angioplasty reduced acute events but neither improved cardiac events at 7 months nor produced changes, compared with heparin, in the minimal luminal diameter at angiographic follow-up.

Desirudin in Acute Coronary Syndromes

Pilot Trials of Desirudin in Unstable Angina without PTCA

In a multicenter open-label pilot trial, 166 patients with unstable angina and angiographic thrombus were randomized to a 72- to 120-hour infusion of heparin (50 patients) or desirudin (116 patients) at 0.05 mg/kg/hr to 0.3 mg/kg/hr (32). Heparin was adjusted to an APTT of 65 to 90 seconds (28 patients) or 90 to 110 seconds (22 patients), whereas desirudin was not adjusted to APTT prolongation. APTT prolongations with desirudin at 0.2 and 0.3 mg/kg/hr were not significantly different. Upon repeat angiography at 72 to 120 hours, patients assigned to desirudin (compared with heparin) had an improved cross-sectional area of the culprit vessel ($p = 0.08$) and a larger minimum cross-sectional area ($p = 0.028$), whereas the improvement in TIMI flow grade was not significant ($p =$

0.44). Equal angiographic benefit was seen with desirudin at 0.1 and 0.3 mg/kg/hr, suggesting a plateau effect for desirudin at mg/kg/hr. Clinical outcomes at 30 days were not significantly different. Myocardial infarction (MI) developed in 2% of desirudin : 8% of heparin patients ($p = 0.11$). No patient died, and none had intracerebral bleeding or another major spontaneous bleed. No reboar activation of angina was observed after withdrawal of desirudin (32).

OASIS-1 (Organization to Assess Strategies for Ischemic Syndromes) is a relatively large pilot study in 368 patients with unstable angina or suspected myocardial infarction without PTCA (33). Patients were randomized to one of two doses of desirudin: mg/kg bolus, 0.1 mg/kg/hr over 72 hours; 0.4 mg/kg bolus, 0.15 mg/kg/hr over hours) or heparin (5,000 IU bolus, 1,200 IU infusion over 72 hours) (14). Overall, 96% of the patients received aspirin. From an efficacy standpoint, at 7 days there was a trend toward lower event rates in the desirudin groups, particularly in the higher hirudin dose. When the two desirudin groups were combined, there was a significant decrease in the combined incidence of death, myocardial infarction, revascularization at 7 days. No cerebral hemorrhages occurred in the study. There was a significant increase in major bleeding events with desirudin, but the incidence of major bleeding events was higher with desirudin particularly in the higher dose group.

These promising initial results need to be confirmed with longer follow-up and in larger randomized studies. Such a study is OASIS-2, which is a 2×2 factorial design testing the effects of a single regimen of hirudin versus heparin and warfarin versus placebo in 8,000 patients with unstable angina. The endpoints are death, myocardial infarction, and refractory angina.

Pilot Trials of Desirudin as Adjunct to Thrombolysis in Myocardial Infarction

In the open-label TIMI 5 pilot study of acute myocardial infarction treated with f

loaded t-PA, aspirin, and either heparin or desirudin, 162 patients received a 5-day infusion of escalating desirudin dosage (0.05 to 0.2 mg/kg/hr). Eighty-four received heparin adjusted to 65 to 90 seconds (34). Although, the difference in TIMI grade 2 and 3 flow was not significantly different between desirudin and heparin at 90 minutes (82.1% versus 78.6%, respectively), it reached significance at 18 to 26 hours (97.8% versus 89.2%, respectively, $p < 0.01$) due to a decrease in reocclusion rates (1.6% versus 6.7%, respectively, in patients receiving desirudin and heparin, $p < 0.07$) and a higher rate of reperfusion in the desirudin group. Major spontaneous hemorrhage occurred in 4.7% of heparin-treated versus 1.2% of desirudin-treated patients. Intracranial hemorrhage occurred in one heparin patient (34).

Patients in the TIMI-6 pilot trial were randomized to streptokinase (SK), aspirin, and desirudin or heparin. Desirudin appeared as safe as heparin, but the higher doses of desirudin (0.3 mg/kg bolus followed by 0.1 mg/kg/hr) was associated with a trend toward lower rates of death, reinfarction, cardiogenic shock, and congestive heart failure (35).

Large-Scale Randomized Multicenter Trials with Desirudin in Patients with Acute Myocardial Infarction

Results of the GUSTO-IIa, TIMI-9A and HIT-III Trials

Because the phase II pilot trials suggested that desirudin and heparin, at the doses tested, were safe, two large-scale heparin controlled studies—TIMI-9A (36) and GUSTO-IIa (37)—were started in patients with acute myocardial infarction with the same brand name of desirudin (RevascTM) and in a third trial coded HIT-III (38) with another recombinant hirudin (LepirudinTM). In the first two, desirudin was administered as a 0.6 mg/kg i.v. bolus followed by a fixed-dose infusion of 0.2 mg/kg/hr for 96 hours (TIMI-9A) or for 72 to 120 hours (GUSTO-IIa). All patients received aspirin. In the much smaller HIT-III trial, pa-

tients were randomized to a 48- to 72-hour infusion of desirudin (HBW023, LepirudinTM) at a dose of 0.4 mg/kg i.v. bolus, followed by an infusion of 0.15 mg/kg/hr or a bolus of 70 IU heparin/kg followed by 15 IU/kg/hr. A front-loaded alteplase protocol was used, and all patients received aspirin; 15% of patients in TIMI-9A received SK. In HIT-III, but not in GUSTO-IIa or TIMI-9A, the dose of desirudin was adjusted to achieve APTT values two to 3.5 times baseline.

When 2,564 patients with acute myocardial infarction had been enrolled in GUSTO-IIa, the trial was halted because intracranial bleeding rates of 0.9% and 1.9% with alteplase (with heparin or desirudin) and the astonishingly high rates of 2.7% and 3.2% with SK (with heparin or desirudin) were up to two times greater than GUSTO-I (0.7%). Similarly, when 757 had been enrolled in TIMI-9A, this trial was suspended because of a high rate of cerebral bleeding (1.9% and 1.7% in patients given alteplase with heparin or desirudin, respectively).

Intracranial bleeding rates in the HIT-III trial were 3.4% in the 154 patients receiving recombinant hirudin but none in the group receiving heparin (38). All hemorrhagic strokes in HIT-III occurred within 24 hours of the start of treatment. In TIMI-9A and HIT-III trials, patients with major bleeding treated with desirudin tended to have higher median APTT values 12 hours after the start of treatment than did those without major bleeding.

In HIT-III, but not in GUSTO-IIa or TIMI-9A, adjustment of the study drug to an APTT prolongation of two to 3.5 times baseline was recommended. In addition to front-loaded t-PA (or SK in 15% of TIMI-9a patients), all patients in these three trials received aspirin. In contrast to GUSTO-I, where 50% of patients had an APTT below the target range of 60 to 85 seconds, a weight-adjusted heparin regimen was used in GUSTO-IIa and TIMI-9A (patients weighting less than 80 kg and ≥ 80 kg, respectively, 1,000 and 1,300 IU/hr) with titration to a target range of 60 to 90 seconds. This strategy resulted in a 20% increase in the total amount of heparin given. Heparin in HIT-III

was weight adjusted (70 IU/kg followed by 15 IU/kg/hr). In TIMI-9A, major spontaneous noncerebral hemorrhage occurred in 7.0% and 3.0%, respectively ($p < 0.02$). A baseline creatinine of more than 1.5 mg/day, older age, lower body weight, and a higher APTT (100 seconds versus 86 seconds in nonstroke patients) were associated with bleeding in desirudin patients, suggesting that reduced renal clearance of desirudin could have contributed to the higher bleeding risk. In GUSTO-IIa, there was a trend toward increased intracerebral bleeding with age, female sex, and greater APTT prolongation (12-hour APTT 110 and 87 seconds, respectively, in patients with and without stroke, $p = 0.031$). This contrasted with the hemorrhagic stroke rates in GUSTO-1 (0.57% with i.v. heparin plus SK and 0.7% with i.v. heparin plus t-PA). The incidence of hemorrhagic stroke in GUSTO-IIa patients not receiving thrombolytic treatment was also relatively high (0.3%, all of which were patients randomized to desirudin). Stroke on thrombolytics occurred at a median time of 8 hours after start with desirudin and after 17 hours with heparin ($p = \text{NS}$).

HIT-III was also stopped when an imbalance in the incidence of hemorrhagic stroke became apparent. The incidence of confirmed cardiac rupture was 2% in desirudin versus 0.6% in heparin patients. Overall, spontaneous bleeding (other than intracranial) occurred in 2.7% of desirudin versus 1.3% of heparin patients. All hemorrhagic strokes (all on desirudin) occurred within the first 24 hours after treatment start. HIT-III patients bleeding on desirudin had a median APTT of 106 seconds versus 76 seconds in those who did not bleed. The early plasma desirudin levels produced by the 0.6 mg/kg bolus appeared to be in excess to what has been predicted in a phase I trial of patients with stable coronary artery disease and normal serum creatinine (39).

Rationale of the Reduced Desirudin and Heparin Doses in GUSTO-IIb and TIMI-9B

In view of these results and of the observation that infusion of desirudin at a dose of 0.1

mg/kg/hr was apparently as effective as high doses in both unstable angina and myocardial infarction in pilot studies (32–35), GUSTO-IIb (40) and TIMI-9B (41) were restarted comparing low anticoagulant doses of desirudin (0.1 mg/kg i.v. bolus of desirudin, followed by 0.1 mg/kg/hr) or heparin (1,000 IU/hr not adjusted to body weight). In addition, both heparin infusion and desirudin infusion were adjusted to a reduced target APTT range of 55–85 seconds (TIMI-9B) (instead of 60 to 85 seconds) and 60 to 85 seconds (GUSTO-II) (instead of 60 to 90 seconds) because APTT values above 100 seconds clearly were associated with increased risk of intracerebral hemorrhage. It was expected that down-titration of desirudin and adjustment to APTT values only two to three times baseline may take better advantage of the lower anticoagulant/antithrombotic ratio of desirudin compared with heparin. This had been clearly established in preclinical studies in which desirudin was more effective than heparin at APTT ratios several times lower than those achieved with high-dose heparin (16,17,19). Perhaps more important for the investigators, the American unstable angina multicenter trial also had suggested a plateau effect for the desirudin dose of 0.1 mg/kg/hr (32).

Results of GUSTO-IIb and TIMI-9B

The results of GUSTO-IIb on 12,142 patients were disappointing. The primary combined endpoint of death or nonfatal myocardial infarction or reinfarction at 30 days was similar in desirudin-treated patients (8.9%) in the heparin group (9.8%, $p = 0.06$). However, at 24 hours the same endpoint was significantly different in favor of desirudin (1.3% versus 2.1%, $p = 0.001$), but this difference did not persist up to 30 days. There were no significant differences in the incidence of serious bleeding (1.2% versus 1.1%, $p = 0.43$), but intracranial bleeding occurred more often in desirudin-treated patients (0.1% versus 0.2%, $p = 0.24$). Desirudin therapy was associated with a significantly higher incidence of moderate bleeding (8.8% versus 7.7%, $p = 0.03$).

The same desirudin brand and dose was used in the 3,002 patients enrolled in TIMI-9B as in GUSTO-IIb. Intravenous desirudin or heparin were administered for 96 hours. The primary endpoint was a 30-day incidence of death, myocardial infarction, congestive heart failure, and shock. In contrast to the results of the GUSTO-IIb trial, there was no significant difference in TIMI-9B in the primary endpoint between either desirudin (12.8%) or heparin (11.8%) or in the incidence of death and myocardial infarction (9.6% versus 9.3%). Similarly, there was no significant difference in major bleeding events (4.6% versus 5.3%) between treatment groups. Intracranial bleeding occurred in 0.4% of the desirudin patients and 0.9% of the heparin patients.

Combined analysis of the results of both megatrials suggests a modest but significant reduction of 13% ($p = 0.026$) in the (re)infarction incidence with desirudin at 30 days (corresponding to an absolute reduction of nine events per 1,000 patients treated) without a striking effect on mortality. Among the 12,142 patients in GUSTO-IIb, 3,457 (28.5%) were treated with thrombolytics. Among them, 3,289 had presented with ST-segment elevation at the time of enrollment. They were treated at the investigator's discretion with either t-PA (2,274 patients) or SK (1,015 patients) and randomized to receive either heparin or hirudin as guided by the main GUSTO-IIb randomization. For the 1,015 patients receiving SK, a marked 40% reduction in the primary endpoint, death/reinfarction at 30 days, was demonstrated in patients treated with hirudin (8.6%) compared with heparin (14.4%) (odds ratio [OR] = 1.78, 95% CI = 1.20 to 2.66, $p = 0.004$). For the patients receiving t-PA ($n = 2,274$), there was only a minor (5.5%) reduction in the primary endpoint (30-day death/myocardial infarction) for accelerated t-PA reduced from 10.9% with heparin to 10.3% with hirudin (OR = 1.06, 95% CI = 0.81 to 1.38; $p = 0.68$, for treatment heterogeneity, $\chi^2 = 4.45$, $p = 0.03$), suggesting a significant treatment effect in outcomes specific for SK-treated patients who were randomized to hirudin rather than heparin (42). Thus, a favorable treatment interaction of SK,

but not t-PA, with hirudin, was demonstrated. These findings, coupled with recent trials combining SK and direct thrombin inhibitors, provide support for the importance of thrombin activity after therapy with this plasminogen activator.

Lessons Learned from the Large-Scale Trials with Desirudin in Patients with Acute Myocardial Infarction

The set dose of desirudin was obviously too high in GUSTO-IIa and TIMI-9A. This could possibly have been predicted from the dose-ranging TIMI-5 trial, where the highest dose of desirudin (0.6 mg/kg bolus followed by 0.2 mg/kg/hr infusion for 120 hours) was associated with a significant risk for major hemorrhage (29.4%) compared with lower desirudin doses (10.9%) (34). Also in TIMI-6 the same high dose of desirudin resulted in a higher rate of major bleeding (29%) compared with the lower doses of desirudin (13%, $p = 0.007$). This highest dose tested in phase II trials was probably selected in GUSTO-2a and TIMI-9A in view of the lack of a dose-response relationship in the three phase II pilot trials.

This decision was also based on the finding in GUSTO-I and in other trials that subtherapeutic anticoagulation was associated with lower infarct-related coronary patency. Indeed, weight-adjusted heparin (more than 80 kg: 1,300 IU/hr) was used in GUSTO-2a and TIMI-9A because approximately 50% of patients receiving i.v. heparin in GUSTO-I had APTTs below the predefined range of 60 to 85 seconds, with the majority of these patients weighing more than 80 kg. Considering the high incidence of cerebral bleeding, the heparin dose also was decreased in GUSTO-IIb and TIMI-9B to lower APTT target values and was not weight adjusted further. Moreover, the infusion of desirudin instead of being fixed was now adapted to the same APTT target values as for heparin (60 to 85 seconds), a range that is lower than in GUSTO-Ia and TIMI-9A (60 to 90 seconds).

The bolus dose of desirudin was drastically decreased from 0.6 mg/kg (in GUSTO-IIa and TIMI-9a) to 0.1 mg/kg in GUSTO-IIb and

TIMI-9B because 39% of the major hemorrhagic events occurred within 24 hours of initiation of thrombolysis and study drug treatment in the former studies; moreover, desirudin-treated patients experience major bleeding earlier in the course of treatment than heparin-treated patients in TIMI-9A (mean 8 hours versus 17 hours). It is possible that the dose of hirudin in GUSTO-IIb and TIMI-9B has been reduced too much to obtain therapeutic efficacy in the clinical endpoints. Of note, in the subsequently reported but not fully published OASIS trial using an intermediate dose of desirudin (a bolus of 0.4 mg/kg followed by an infusion of 0.15 mg/kg/hr), the incidence of bleeding was twice as high in the desirudin group as in the heparin group (43). The latter trial was conducted in another group of patients (unstable angina and suspected myocardial infarction), with another brand of recombinant hirudin (HBW 023) and in the absence of thrombolytic treatment. This suggests that there is a narrow therapeutic window for recombinant hirudin.

Another explanation for the disappointing therapeutic results of GUSTO-IIb and TIMI-9B could be the short duration of hirudin infusion. In TIMI-9B all the beneficial results were evident within the first 24 hours; beyond that point, the event-rate curves neither diverge nor converge. For equivalent fibrinopeptide A levels (reflecting thrombin activity), a greater reduction in prothrombin fragment 1+2 (F1+2) levels (reflecting thrombin generation) is seen with heparin compared with desirudin, even at high doses (2,39,43,44). In contrast, for equivalent F1+2 levels, a greater reduction in fibrinogen peptide A (FPA) levels is seen with hirudin compared with heparin (45). Thus, hirudin at medium and high doses has a relatively smaller effect than heparin in the feedback-amplifying mechanisms leading to prothrombinase generation and thrombin formation. The accumulation of thrombin at a thrombogenic surface (ruptured coronary plaque) may have detracted from the ability of desirudin to inhibit the activity of thrombin. Although the

proposal of a longer infusion of desirudin is attractive, the lack of clinical benefit observed in the TIMI-9A trial, in which anticoagulation was administered an average 30% longer than in TIMI-9B (120 hours versus 96 hours), makes this possibility less probable.

A third possibility is the rebound hypercoagulability noted after cessation of antithrombotic therapy in patients with coronary syndromes as observed after withdrawal of argatroban, heparin, bivalirudin, and desirudin.

A fourth possibility is that mixing patients with and without ST elevation may not be appropriate because the mortality rate in patients without ST elevation at 24 hours about one quarter of that of patients with ST elevation.

Because of the worse performance of high dose desirudin, compared with heparin, as an inhibitor of thrombin generation (10–13,42,4) and its failure to inhibit the wave of thrombin generation triggered by thrombolytics (4), additional interventions blocking thrombin generation, including heparin itself, factor X inhibitors, or inhibitors of tissue factor, may be required to further address this issue. Nonetheless, as earlier mentioned, the potential for the direct thrombin inhibitors to induce activation of protein C may represent another inherent limitation of all members of this class of drugs. Furthermore, the IIb/3 platelet receptor antagonist does induce moderate inhibition of thrombin generation reflected, in the case of abciximab (ReoPro, Eli Lilly and Company, Indianapolis, IN), in prolongation of the ACT by about 35 seconds when used during percutaneous revascularization procedures (46). To a mild degree this is also true of aspirin (47). Experimental evidence also indicates that combined administration of relatively low doses of platelet receptor and thrombin inhibitors may be efficacious (48). Whether such combinations will be tested in clinical trials in the future remains to be seen, especially in view of the high recurrent event rates with present antithrombotic regimens of thrombin inhibitors (b

heparin and desirudin), aspirin, and plasminogen activators.

Desirudin in Deep Vein Thrombosis

Recombinant desirudin has been used for the prevention and treatment of venous thromboembolism.

Desirudin was evaluated in two consecutive studies performed in patients having total hip replacement. The aim was first to find the optimal dose of desirudin (10, 15 or 20 mg s.c. twice daily) in comparison with unfractionated heparin (5,000 IU s.c. three times daily) (49) and second to investigate whether the efficacy and safety of desirudin (15 mg s.c. twice daily) could compete with a low molecular weight heparin (enoxaparin, 40 mg daily) in patients undergoing total hip replacement. Both trials were prospective, randomized, and double-blind and all regimens were started preoperatively and trial drugs administered s.c. for 8 to 12 days. The main efficacy parameter was the presence of deep venous thrombosis verified by mandatory bilateral phlebography at the end of the prophylaxis period. The phlebography was evaluated centrally. Safety was mainly evaluated by blood loss and transfusion requirements. In the first study the rates of proximal deep venous thrombosis in 1,120 patients were 19.6% for unfractionated heparin and 8.5%, 3.1%, and 2.4% for desirudin 10 mg, 15 mg, and 20 mg, respectively ($p < 0.01$).

The 15-mg dose of desirudin provided the best benefit/risk ratio and was selected for the next investigation in 2,079 patients with total hip replacement. The incidence of proximal deep venous thrombosis was 7.5% and 4.5% in the enoxaparin and the desirudin groups, respectively ($p < 0.02$) with a relative risk reduction of 40%. The safety profiles of the regimens were comparable. The results from this trial shows that the desirudin provides a benefit/risk ratio superior to that of low-molecular-weight heparin.

Two small pilot trials of desirudin for the treatment of established deep vein thrombosis failed to show significant changes in lower

limb venography after 5 days of treatment (50,51). The performance of desirudin versus low molecular weight heparin in the prevention and treatment of thromboembolism has not been tested in clinical trials.

Desirudin in Heparin-Induced Thrombocytopenia

Immune-mediated heparin-induced thrombocytopenia, an uncommon and severe complication of heparin therapy, may be associated with venous and arterial thromboembolism. Desirudin has been used in a small trial of heparin-induced thrombocytopenia as an alternative to heparin, with resolution of thrombocytopenia and clinical complications (52).

Neutralization of Desirudin

Bleeding with hirudin should prompt immediate arrest of the desirudin infusion, and consideration should be given to the administration of DDAVP, a vasopressin analogue. Experimentally, DDAVP shortens the APTT and bleeding time prolonged by desirudin (53–55). In human volunteers, DDAVP, when given over 15 minutes in a dose of 0.3 $\mu\text{g/kg}$, has been shown to reduce the prolongation of APTT after desirudin administration (56). Prothrombin complex concentrate has been shown to reduce the bleeding response to desirudin, but cannot be recommended at this time. When bleeding is life threatening, consideration should be given to hemodialysis.

HIRUGEN

Modelled on the C-terminal fragment of hirudin, hirugen is a synthetic dodecapeptide comprising the 12 terminal residues of hirudin that block the fibrinogen binding site (the anion-binding exosite) of thrombin; the molecule contains sulfated tyrosine to increase its thrombin affinity (Fig. 9-2). Hirugen (BG 8863) inhibits thrombin, forming an inhibitor complex of substantially lower affinity (about 50 times) compared with hirudin (K_D of 1.5×10^{-7} mol/L versus 0.2×10^{-12}

mol/L for r-hirudin). In vitro, hirugen competitively inhibits thrombin-mediated fibrinogen cleavage and platelet activation (57,58). Because it does not block the active site of thrombin, hirugen does not block thrombin mediated by dialysis of low molecular weight synthetic substrates.

In experiments with exteriorized arteriovenous shunts in baboons, hirugen prevented ex vivo platelet deposition in low-shear flow chambers connected to chronic arteriovenous shunts of baboons but failed to affect ex vivo platelet deposition on collagen type I-coated tubing at a dose of 75 mg/kg (APTT fourfold baseline) (59).

Presently, no clinical studies with hirugen are underway because its antithrombotic activity is much less potent compared with hirudin and with the follow-up molecule hirulog.

BIVALIRUDIN

Coupling of peptides that mimic the carboxyterminal of hirudin to peptides that are specific for inhibition of the catalytic site of thrombin (D-Phe-Pro-Arg) has led to the development of a chimeric molecule termed bivalirudin (BG 8967, brandname Hirulog, Biosen, Cambridge, MA), in which the amino-terminus consists of the catalytic site-directed tetrapeptide, whereas the carboxy-terminus consists of the 12 terminal residues of hirudin. The two moieties are linked together by a bridge of glycine residues of variable length, and the whole molecule comprises 20 amino acids (60,61). Thus, bivalirudin inhibits thrombin by binding to both its catalytic site and its anion-binding exosite, conferring specificity to these molecules for thrombin. Its K_D toward thrombin is 2.3×10^{-9} mol/L (Fig. 9-2). Bivalirudin is a direct and specific inhibitor of free (fluid phase) and clot-bound thrombin. The hirulog-thrombin complex is only transient because thrombin, once complexed, can slowly cleave the Arg₃-Pro₄ bound on the N-terminal extension with catalytic rate constant (k_{cat}) = 0.012 seconds⁻¹. This metabolic cleavage contributes to its short half-life on the APTT

of about 23 to 36 minutes (62,63). Only 20% of hirulog is excreted in the urine, indicating an extensive hepatic catabolism or proteolysis at other sites. Newer noncleavable bivalirudins have been synthesized containing a homoarginine at the scissile bond. As for other direct thrombin inhibitors, there is no antidote for bivalirudin.

In animal models of venous thrombosis, arterial thrombosis, and thrombolysis, bivalirudin demonstrated greater antithrombotic activity than heparin (64-68).

Pharmacodynamics and Dose-Finding Studies

Phase I studies in healthy volunteers show a dose-dependent prolongation of the APTT with a 15-minute i.v. infusion of bivalirudin 0.05 to 0.6 mg/kg, resulting in APTTs from 1 ± 0.08 to 2.8 ± 0.55 times baseline (3). When 0.3 mg/kg/hr of bivalirudin was infused during 12 or 24 hours, peak APTT ratios were 2.1 to 2.5. There was a good, although not linear, correlation between APTT and bivalirudin plasma concentrations. In turn, there is a linear relationship of total bivalirudin dose administered to the effect area for its anticoagulant activity. Thrombin times (too sensitive) and prothrombin times (not sensitive enough) were not useful in titrating the dose of bivalirudin. After infusion, the half-life was 24 minutes with volume of distribution of 13.0 and a clearance rate of 419 ± 37 ml/min (63). There is no measurable effect of aspirin on bivalirudin anticoagulant activity, and bivalirudin does not alter the effect of aspirin on template bleeding time.

In a dose-finding study in 45 patients undergoing routine cardiac catheterization, good correlation was confirmed between APTT and plasma bivalirudin levels ($r = 0.7$) (69). The APTT was prolonged to 1.8 and 2.5 times baseline, respectively, 15 minutes after starting i.v. bivalirudin at 0.05 mg/kg (bolus) followed by 0.2 mg/kg/hr and 0.15 mg/kg/hr followed by 0.6 mg/kg/hr. No major hematoma or thrombotic complications occurred at both doses. FPA levels were suppressed during the hirulog administration.

doses that, compared with heparin, caused less elevation in APTT, PT, and ACT.

In a dose-escalating pilot study, Lidon et al. (70) evaluated bivalirudin in 55 patients with unstable angina who also received aspirin and triple antiischemic therapy. Bivalirudin was administered in escalating dosages of 0.02 to 0.5 mg/kg/hr, increased every 30 minutes for 72 hours. With dosages up to 1 mg/kg/hr, only one patient of 20 experienced recurrent chest pain. The APTT in angina-free patients averaged 55.6 ± 6 seconds. Plasma FPA levels were suppressed at dosages of 0.25 to 0.5 mg/kg/hr. The APTTs decreased to baseline 4 hours after discontinuation of hirulog. There was no rebound elevation of FPA at that time. Neither interaction with i.v. nitrates nor a cumulative effect were noted when bivalirudin was administered for up to 5 days.

The TIMI-7 pilot trial was designed to evaluate whether a dose response existed in the efficacy of bivalirudin in conjunction with aspirin in patients with unstable angina (71). Four hundred ten such patients were randomized to receive i.v. bivalirudin 0.02, 0.25, 0.5, or 1 mg/kg/hr for 72 hours, in addition to oral aspirin (325 mg/day). There were no significant differences between the different dose levels for the occurrence of the primary efficacy composite endpoint "unsatisfactory outcome" (death, nonfatal myocardial infarction, recurrent ischemia pain at rest with ECG changes, or rapid clinical deterioration necessitating emergency angiography/revascularization within 72 hours), which occurred in 6.2% to 11.4% of patients in each group. However, nonfatal myocardial infarction or death during hospitalization occurred in significantly fewer patients who received one of the three higher doses of bivalirudin compared with those who received 0.02 mg/kg/hr (3.2% versus 10% of patients, respectively), this difference being still present at 6 months' follow-up. Bivalirudin was investigated as an adjunct to thrombolysis with the goals of accelerating drug-induced thrombolysis and to prevent thrombus progression and vessel reocclusion. Lidon et al. (72) randomized 45 patients to bivalirudin (0.5 mg/kg/hr without

prior bolus, reduced to 0.1 mg/kg/hr after 12 hours) or heparin (1,000 IU/hr) added to SK. At 90 and 120 min, TIMI grade 2 and 3 flow was observed in 77% and 87% of patients treated with bivalirudin, respectively. TIMI grade 3 flow was present at 120 min in 77% of bivalirudin versus 40% of heparin patients. In patients receiving heparin plus SK, the corresponding rates of TIMI 2 and 3 flow were 47% for both time points ($p < 0.05$ for the 90-minute point and $p < 0.01$ for the 120-minute point). Bleeding complications occurred in 12% of bivalirudin recipients versus 27% of heparin recipients (no significant difference). There was only one intracerebral hemorrhage, which occurred in the heparin group. APTTs peaked at three and four times baseline, respectively, with bivalirudin and heparin, probably secondary to the fibrinolytic effect of SK, as plasma drug levels were not higher than predicted from phase I studies.

In another pilot study, angiographic patency of the culprit coronary artery lesion was assessed 90 and 120 minutes after the initiation of SK and aspirin and again after 4 ± 2 days in 68 patients with acute myocardial infarction (73). Patients were randomized to bivalirudin 0.5 mg/kg/hr for 12 hours followed by 0.1 mg/kg/hr (low dose), bivalirudin 1 mg/kg/hr for 12 hours then placebo (high dose), or to heparin 5,000 IU bolus then 1,000 IU/hr titrated to an APTT two to 2.5 times control after 12 hours. At 90 minutes, TIMI grade 2 or 3 was observed in 96% of low-dose bivalirudin recipients versus 79% of high-dose bivalirudin and 46% of heparin recipients ($p = 0.006$). Respective TIMI 3 flow grade rates were 85%, 61%, and 31% of patients ($p = 0.008$). At 120 minutes, respective TIMI 2 or 3 rates were 100%, 82%, and 62% ($p = 0.046$), and TIMI 3 rates were 92%, 68%, and 46% ($p = 0.014$). At 90 minutes the relative risk for restoring TIMI flow grade 3 was 2.77 with low-dose bivalirudin compared with heparin ($p < 0.001$) and 1.4 compared with high-dose bivalirudin ($p = 0.04$). Patients who received a placebo infusion after 12 hours experienced more clinical events and reocclusion during the following 4 days than

did patients in the other groups. In this trial, bivalirudin yielded higher patency rates when used in conjunction with SK and aspirin in the early phase of acute myocardial infarction. High bivalirudin doses are unnecessary and may be less effective than lower doses. This suggests that too much thrombin inhibition may be harmful.

Therapeutic Trials in Patients Undergoing PTCA

In a multicenter trial, Topol et al. evaluated bivalirudin in 291 patients undergoing elective angioplasty and pretreated with aspirin (74). After bolus administration, a 4-hour infusion of bivalirudin at 0.6 to 2.2 mg/kg/hr was given. The results show a dose-dependent effect of bivalirudin toward reduction of acute complications (death, fatal evidence for abrupt closure) in that patients receiving one of the three lower doses had 10.2% acute complications compared with 3.3% in patients receiving the two higher doses of bivalirudin. Although there was a trend toward a dose-related increase in APTT prolongation, there was a wide overlap between APTT of different doses. No statistically significant ACT level was associated with complete prevention of acute coronary closure. There was no prolongation of the bleeding time, and no patient developed life-threatening bleeding. Acute closure within 24 hours was inversely related to the hirulog dose and was 3.9% for the 1.8- and 2.2-mg/kg/hr dose combined.

In a much larger Hirulog Angioplasty Study, bivalirudin at high dose (1.0 mg/kg i.v. bolus followed by a 4-hour infusion at 2.5 mg/kg/hr and a 14- to 20-hour infusion at 0.2 mg/kg/hr) was compared with heparin (1,750 U/kg bolus followed by an 18- to 24-hour infusion of 15 IU/kg/hr) during coronary angioplasty for unstable or postinfarction angina in 4,098 patients, all on aspirin (75). Bivalirudin did not significantly reduce the incidence of the primary composite study endpoint (death, in-hospital death, myocardial infarction, abrupt vessel closure, or rapid deterioration of cardiac origin) (11.4% versus 12.2% for he-

parin) but did result in a lower incidence of bleeding (3.8% versus 9.8%, $p < 0.001$). In prospectively stratified subgroup of 704 patients with postinfarction angina, bivalirudin therapy resulted in a lower incidence of the primary endpoint (9.1% versus 14.2%, $p = 0.04$) and a lower incidence of bleeding (3.0% versus 11.1%, $p < 0.001$), but in a similar cumulative rate of death, myocardial infarction, and repeated revascularization in the months after angioplasty (20.5% versus 25.1%, $p = 0.17$).

Of the 4,098 patients of this trial, 573 had angiographic evidence of coronary thrombus (filling defect, ulcerations, or occlusion). Patients with thrombus had higher rates of abrupt closure (13.4% versus 8.4%, $p = 0.001$) and myocardial infarction (5.1% versus 3.2%, $p = 0.03$) than did those without thrombus. The incidence of myocardial infarction and abrupt vessel closure was identical with both anticoagulants in patients with thrombus-containing lesions (76). This clinical analysis suggests that the direct thrombin inhibitor bivalirudin is equivalent to high-dose heparin for thrombus-containing lesions assessed by angiography.

The Hirulog Early Reperfusion/Occlusion (HERO) trial evaluated two doses of bivalirudin with heparin in 412 patients with acute myocardial infarction treated with aspirin (43). Heparin, 5,000 IU bolus followed by 1,000 to 1,200 IU/hr titrated to APTT, or low-dose bivalirudin (0.125 mg/kg bolus followed by 0.25 mg/kg/hr for 12 hours then 0.1 mg/kg/hr) or high-dose bivalirudin (0.25 mg/kg bolus followed by 0.5 mg/kg/hr for 12 hours then 0.25 mg/kg/hr) were administered in a random fashion. TIMI grade 3 flow at 120 minutes was achieved in 35% of heparin patients and in 48% of the high-dose bivalirudin patients ($p = 0.03$). Of patients who presented within 6 hours of symptom onset, 40% achieved a TIMI-3 flow at 90 minutes, 49% with low-dose bivalirudin, and 50% with high-dose bivalirudin. Among those treated within 3 hours of symptom onset, death, cardiogenic shock, or recurrent myocardial infarction had occurred in 17.9%

the heparin patients and 14% of the low-dose bivalirudin patients at 35 days. Major bleeding occurred in 28% of heparin patients, 14% of low-dose bivalirudin patients, and 19% of high-dose bivalirudin patients. A large-scale randomized trial is now planned.

Thus, high-dose bivalirudin is just as effective as high-dose heparin in preventing ischemic complications in patients who underwent PTCA for unstable angina, but it carries a 60% lower risk of bleeding. Bivalirudin, as compared with heparin, reduces significantly the risk of immediate ischemic complications after PTCA in patients with postinfarction angina, but this difference was no longer apparent after 6 months. Bivalirudin appears to be more effective than heparin in promoting early patency in myocardial infarction patients treated with SK, without increase in risk of bleeding.

Trials for the Prevention and Treatment of Deep Venous Thrombosis

The effect of bivalirudin on the production of prothrombin fragments 1 and 2 was studied in patients with calf vein thrombosis. A single injection, either 1 mg/kg s.c. or 0.6 mg/kg as a 15-minute i.v. infusion induced an incomplete and temporary suppression of prothrombin F1+2 (77). Five dosage regimens of s.c. bivalirudin were tested in the prevention of postoperative venous thrombosis after hip or knee surgery: 0.3 mg/kg every 12 hours, 0.6 mg/kg every 12 hours, 1.0 mg/kg every 12 hours for 3 days followed by 0.6 mg/kg every 12 hours for up to 11 days, 1.0 mg/kg every 12 hours, and 1.0 mg/kg every 8 hours (78). One hundred seventy-seven patients who had technically adequate bilateral venography or objectively documented pulmonary embolism were included in the primary analysis of efficacy. The highest dosage regimen (1.0 mg/kg every 8 hours) provided the lowest rates of total (17%) and proximal deep vein thrombosis (2%), both of which were significantly lower ($p = 0.010$ and $p = 0.023$, respectively) than the pooled rates of total (43%) and proximal (20%) deep vein thrombosis seen with the

first four regimens. Bleeding rates were low (less than 5%) with all regimens. This study demonstrates that 1.0 mg/kg bivalirudin every 8 hours started postoperatively is potentially efficacious and safe for the prevention of deep vein thrombosis after major hip or knee surgery.

HIRUNORM

Several peptide analogues of bivalirudin have been synthesized addressing the very issue of metabolic stability through modification of the amino acid composition of the spacer arm or of the NH_2 -terminus, and by rendering nonhydrolyzable the critical peptide bond. In vitro activity and stability data for these compounds have been published (81,82). This novel class of specific thrombin inhibitors termed "hirunorms" is the result of a different strategy to the same target, that is, to imitate as far as possible hirudin's tridimensional approach to the sites on thrombin surface avoiding the characteristics of a partial substrate proper to hirulog-1 and analogues (83).

Hirunorm is a 26-amino acid computer-modeled synthetic peptide (84). It is equipotent to bivalirudin and 1/30 as potent as desirudin in blocking α -thrombin amidolytic activity ($\text{IC}_{50} = 10 \pm 2$, 15 ± 1 , and 0.3 ± 0.1 nmol/L, respectively), but it does not affect trypsin, plasmin, and t-PA activities at 10 $\mu\text{mol/L}$. Hirunorm inhibits clot-bound thrombin to clots prepared by thrombin hydrolysis of purified fibrinogen in buffer. Hirunorm and hirulog show similar species-dependent potency in doubling basal in vitro clotting times of human, rat, and rabbit plasma (EC200 varied 70 to 200 nmol/L for TT, 0.7 to 16 $\mu\text{mol/L}$ for APTT, and 0.8 to 17 $\mu\text{mol/L}$ for PT), whereas desirudin was always at least three times more active. Hirunorm was stable against α -thrombin and plasma hydrolases, but it was catabolized by rat liver and kidney enzymes.

Venous thrombosis was produced in anesthetized rats by vena cava ligation after a procoagulant serum injection. Intravenous and

subcutaneous hirunorm inhibits venous thrombosis at doses (≥ 0.3 mg/kg) three times higher than those of r-hirudin. Bivalirudin was as active as hirunorm only after i.v. infusion. Arterial thrombosis was obtained in the anesthetized rat by chemical (FeCl_2) stimulation of a common carotid; an i.v. infusion of hirunorm (1 to 3 mg/kg/30 min) inhibited it dose-dependently; desirudin was partly active only at 3 mg/kg, but hirulog was inactive at either dose. Full antithrombotic doses of hirunorm did not affect the bleeding time as measured from punctured mesenteric vessels in anesthetized rats. This compound appears to be a potent peptide thrombin inhibitor endowed with antithrombotic activity in models of venous and arterial thrombosis.

ARGATROBAN

Argatroban (Argipidine, MD-805), a small-molecule derivative of the amino acid L-arginine, was designed to inhibit thrombin directly. Its molecular weight (circa 526 daltons) is considerably smaller than hirudin (circa 7,000 daltons) and low molecular weight heparins (4,500 to 6,500 daltons). This arylsulfonylarginine interacts selectively with serine and the basic pocket of the catalytic site of thrombin along with an adjacent hydrophobic site known as the apolar region of thrombin (Fig. 9-2); concentrations three to four orders of magnitude higher are required to inhibit other serine proteases (Table 9-1). This binding of argatroban to thrombin is rapid at a diffusion controlled rate (85). In contrast with the binding to thrombin of hirudin, which is extremely tight and irreversible (dissociation constant $K_D = 2.3 \times 10^{-13}$ mol/L), the binding of argatroban to thrombin is fully reversible (dissociation constant $K_i = 3.9 \times 10^{-8}$ mol/L) (86).

Argatroban (brandname Novastan or Slonon) is a 64:36 mixture of 21-(R) and 21-(S) diastereoisomers, with the latter being approximately twice as potent as the former in an in vitro coagulation assay but considerably less soluble in aqueous buffer (87).

TABLE 9-2. Inhibition of serine proteases by argatroban

For enzyme	K_D (μM)
Thrombin (human)	0.039
Thrombin (bovine)	0.019
Trypsin	5.0
Factor-Xa	210
Plasmin	800
Kallikrein	1,500

K_D , dissociation constant.

In Vivo Antithrombotic Studies in Experimental Models

Argatroban has been shown to be superior to heparin in erythrocyte-rich and platelet-rich thrombosis in several species of arterial thrombosis when administered as an i.v. bolus or as continuous i.v. infusion (88). Compared with heparin, argatroban is significantly more effective in the prevention of platelet-rich thrombi after vascular injury and was effective at APTTs of only two to three times baseline control (89).

In a whole-blood thrombolysis study with stenosed femoral arteries in the rabbit, argatroban (100 $\mu\text{g/kg/min}$, APTT 2.5- to 3.0-fold baseline) accelerated reperfusion compared with heparin (200 IU/kg, APTT more than fivefold baseline) to the extent of causing a significant leftward shift of the t-PA dose-response curve. Addition of aspirin did not accelerate thrombolysis by either argatroban or heparin (89).

In a whole-blood clot thrombus model with stenotic canine coronaries, pretreatment with argatroban at 200 $\mu\text{g/kg/min}$ (APTTs of six to seven times control) significantly reduced time to lysis by alteplase to 23 minutes compared with 40 minutes in the aspirin group. Addition of aspirin to argatroban did not shorten time to lysis but reduced the incidence of reocclusion by platelet-rich thrombi from 75% to 20% relative to argatroban alone (90). Argatroban was as effective in this model as abciximab in inhibiting the platelet glycoprotein (GP)IIb/IIIa receptor.

In a platelet-rich coronary thrombus model after endothelial injury created by electric current, acceleration of lysis by alteplase was observed in dogs pretreated with argatroban at a lower dose (41 $\mu\text{g/kg/min}$). However, abolition of cyclic flow reductions due to intermittent platelet aggregates required the addition of a thromboxane A_2 /prostaglandin endoperoxide receptor antagonist (91). Of note, in an open-chest canine model of unstable angina, both argatroban and heparin were equally efficacious at abolishing cyclic flow reductions caused by the formation or dislodgement of platelet-rich, fibrin-poor thrombi in the absence of platelet inhibitors (92,93).

The dose of argatroban that doubled the bleeding time (rat-tail transection) was five times greater than for heparin (11 versus 2.2 $\mu\text{g/kg/min}$) (94).

Overall, in experimental models of arterial thrombosis, argatroban achieves *in vivo* antithrombotic efficacy comparable with that of heparin, but with less systemic anticoagulation (APTT) and hemorrhagic potential.

In the Wessler venous thrombosis model (thromboplastin plus stasis of the left jugular vein) and arteriovenous shunt models in rabbits, argatroban was less active on a weight basis than heparin (95).

Pharmacokinetics and Pharmacodynamics

In rabbits and dogs, radiolabeled argatroban is cleared from the plasma in a biphasic manner, with α and β elimination half-lives of 3 to 6 minutes and 20 to 86 minutes, respectively (96). In normal volunteers, the elimination half-life is around 30 minutes. The majority of the drug is excreted fecally, indicating hepatic metabolism and biliary excretion (96). The metabolism is hydroxylation and aromatization of the 3-methyltetrahydroquinoline ring (97).

Compared with heparin, bolus doses of argatroban showed a slowly increasing dose-response effect in normal subjects, with an eightfold increase in dose (30 to 240 $\mu\text{g/kg}$) resulting in only a twofold increase in peak

APTT (43 to 82 seconds). However, heparin showed a pronounced, rapidly rising effect on APTT, with a twofold increase in dose (15 to 30 IU/kg) doubling the peak APTT value (68 to 269 seconds) and with doses ≥ 30 IU/kg often associated with APTT values of more than 400 seconds, which is above the assay detection limits (98).

Figure 9-3 compares the effects of 4-hour infusions of various doses of argatroban or heparin on APTT. Over a twofold increase in dose, heparin displayed a steep dose-response curve; argatroban, however, displayed a gently rising, predictable response over an eightfold range in infusion dose (98).

Figure 9-4 compares the time course of a combined bolus and continuous *i.v.* infusion regimen (4-hour duration) for argatroban (250- $\mu\text{g/kg}$ bolus and 10- $\mu\text{g/kg/min}$ infusion) and heparin (125-U/kg bolus and 0.3-U/kg/min infusion) on HemoTec ACT (98). Both drugs rapidly induced increases in ACT over baseline values. Argatroban maintained the ACT values at steady levels for 4 hours; the response with heparin, however, decreased during the infusion, likely a result of

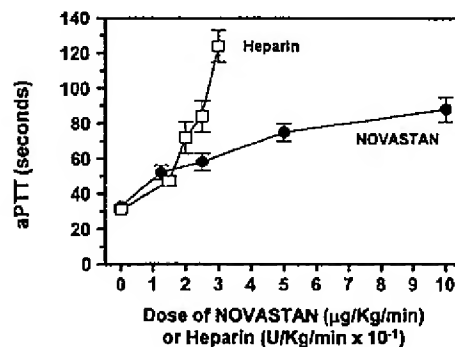


FIG. 9-3. Pharmacodynamic effects of continuous infusions (4-hr duration) of heparin (open squares) and argatroban (filled circles) on the mean APTT (\pm SEM) in nine normal subjects. The doses used were as follows: argatroban, 1.25, 2.5, 5.0, and 10.0 $\mu\text{g/kg/min}$; heparin, 0.15, 0.20, 0.25, and 0.30 IU/kg/min. (From Schwartz et al., ref. 98, with permission.)

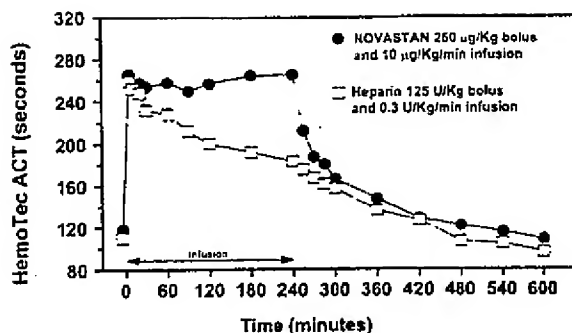


FIG. 9-4. Comparative effects of combined bolus injection continuous infusion (4-hr duration) of heparin (open squares) or argatroban (filled circles) on the mean HemoTec ACT for nine normal subjects. (From Schwartz et al., ref. 98, with permission.)

the release of platelet factor 4 from activated platelets, which interferes with the binding of heparin to antithrombin III.

The effects of renal impairment, age, and gender on the pharmacodynamics of argatroban have been studied. No significant effects were observed, but there was a trend toward an increased risk for bleeding events in patients with an APTT of more than 90 seconds, particularly in elderly, low body weight patients (especially female patients), and patients with renal impairment (99,100).

Clinical Development

The first clinical pilot study with argatroban (Novastan; Mitsubishi Kasai Corp., Midori-ku, Yokohama, Japan) was in 43 patients with unstable angina/non-Q wave myocardial infarction. Argatroban was infused over 4 hours (0.5 to 5.0 µg/kg/min), which resulted in a dose-dependent increase in APTT and effectively prevented recurrences of ischemic episodes, in the absence of aspirin (101). However, it was reported that cessation of therapy was associated with "rebound" thrombin generation (as measured by levels of plasma thrombin-antithrombin complex (TAT) and with an early dose-related recurrence of unstable angina. One should remember that abrupt termination of a thrombin inhibitor increases thrombin activity (as measured by FPA formation) but not thrombin

generation (as measured by the formation of either TAT or prothrombin fragment F1.2). The short duration of the argatroban infusion (4 hours) may be criticized for a condition in which the tendency toward thrombosis remains for days (102). No decrease in plasma TAT concentration was observed during the argatroban infusion, questioning whether the TAT increase observed after cessation of argatroban therapy is artifactual and no elevation in plasma FPA was observed after cessation of therapy, as would be expected in a hypercoagulable state (103). However, it is also possible that the dissociation of the drug from thrombin (argatroban is a competitive inhibitor) allows regeneration and a rebound of thrombin (104). It remains to be established in larger trials whether argatroban offers a favorable clinical profile in unstable angina and non-Q wave myocardial infarction.

There is limited published experience with argatroban in PTCA patients. In order to define the optimal dose, 30 patients undergoing PTCA for stable or unstable angina were studied at four different dose regimens (105). Study endpoints were the occurrence of clinical cardiac events, bleeding complications, coagulation tests, and qualitative angiographic interpretation. All patients underwent control angiography 18 to 24 hours after PTCA. The group with the highest argatroban dosage received 250 µg/kg i.v. bolus followed by a 4-hour infusion of 15 µg/kg/min. At 4 hours

infusion rate was lowered to 3.8 $\mu\text{g/kg/min}$ and continued for 68 hours. TT, APTT, and PT were significantly related to argatroban plasma levels (R-square 0.64, 0.71, 0.84 by regression analysis, respectively). F1+2 and TAT did not relate to argatroban plasma levels. Five patients experienced a cardiac event, and there were two cases of prolonged bleeding at a puncture site, one false aneurysm, and one epistaxis occurring under heparin and acenocoumaron 3 days after stopping argatroban requiring transfusion. This pilot trial identified an apparent safe and adequate dose regimen that is presently being evaluated in a double-blind, 2:1 randomized comparative trial versus heparin (Argaplasty trial).

In a pilot trial, i.v. argatroban was assessed versus unfractionated heparin as adjunctive therapy to the accelerated regimen of alteplase in 112 patients with acute myocardial infarction (106). Argatroban was given as a bolus of 100 $\mu\text{g/kg}$ before the start of thrombolytic therapy followed by an infusion of 3 $\mu\text{g/kg/min}$. Heparin was administered as a bolus of 5,000 IU followed by an infusion of 1,000 IU/hr titrated against APTT. The TIMI grade 3 patency rate at 90 min was 56% in the argatroban group and 67% in the heparin group, a nonstatistical difference. Larger trials are required to decide the optimal dosage of argatroban and to assess its angiographic and clinical efficacy compared with unfractionated heparin.

Two multicenter, randomized, blinded, controlled clinical trials are assessing the efficacy and safety of argatroban as adjunctive therapy to thrombolytic agents in the treatment of acute myocardial infarction. The Argatroban in Myocardial Infarction (AMI) study has enrolled 400 patients with a diagnosis of myocardial infarction within the first 6 hours of onset of symptoms. All patients received aspirin and SK and were randomized to receive either high-dose argatroban (3.0 $\mu\text{g/kg/min}$), low-dose argatroban (1.0 $\mu\text{g/kg/min}$), or placebo for 48 to 72 hours. The dose of argatroban is to be titrated downward if the APTT exceeds 90 seconds. Patients were followed for 30 days to assess the incidence of death, acute myocardial infarction, recurrent angina,

need for coronary revascularization procedures (PTCA or CABG surgery), and new-onset congestive heart failure. In a substudy of an additional 180 patients, patency of the culprit coronary artery was assessed angiographically at 90 and 120 minutes after initiation of therapy.

A second trial, the Myocardial Infarction with Novastan and t-PA (MINT) Study was terminated. One hundred twenty patients were enrolled with the same diagnostic criteria as in the AMI trial. All patients received aspirin and accelerated t-PA and were randomized to one of three groups: (a) high-dose argatroban (3.0 $\mu\text{g/kg/min}$), (b) low-dose argatroban (1.0 $\mu\text{g/kg/min}$), or (c) heparin for 48 to 72 hours. As in the AMI study, patency of the culprit coronary artery was assessed angiographically at 90 and 120 minutes after initiation of therapy.

One pivotal multicenter trial investigating the safety and efficacy of argatroban in patients with heparin-induced thrombocytopenia (HIT) has been completed.

EFEGATRAN

Efegatran sulfate (GYKI 14766, LY 294468), a tripeptide aldehyde (mePhe-Pro-Arg-H) is an arginal catalytic-site inhibitor of thrombin (107). It is a reversible, competitive, tight-binding inhibitor (108). No time-dependent effects were observed for interactions of efegatran with thrombin, suggesting that there are no slow-binding interactions of practical consequence.

Table 9-3 shows that a concentration of 20 ng/ml of efegatran is required to double the TT, but over 1,000 ng/ml is required to prolong the PT and APTT. The functional anticoagulant selectivity for an inhibitor can be estimated by a ratio of the concentrations that prolong by twofold the APTT and the TT; such APTT/TT effect ratios are shown in Table 9-3. For efegatran the APTT/TT ratio is 55, which means that 55-fold higher concentrations are required for APTT prolongation (109).

These data suggest that hirudin and efegatran, although both are direct-acting inhibitors of thrombin, act differently on the APTT

TABLE 9-3. Human plasma anticoagulant concentration for two-fold prolongation of thrombin time, prothrombin time, and APTT

	Thrombin time		Prothrombin time (ng/ml)	APTT (ng/ml)	Ratio of APTT/TT
	ng/ml	nmol/L			
Efegatran	19 ± 2	33	1,360	1,050	55
Native hirudin	109 ± 4	16	1,800	280	2.5
Recombinant hirudin	126 ± 17	18	3,600	340	2.7

Control TT, 32 seconds; control PT, 18 seconds; control APTT, 32 seconds. From Smith et al., ref. 109, with permission.

pathway, possibly by inhibiting a different APTT element in addition to thrombin inhibition, or by differently affecting a thrombin-mediated function in the APTT pathway. Hypothetically, different effects on the thrombin feedback activation of factor V or factor VIII could cause the observed anticoagulant differences. More speculatively, if thrombin activation of protein C should prove to be an element of the APTT pathway and clotting rate, then different inhibition of this process by hirudin and efegatran could cause the observed APTT/TT selectivity differences. However, the observed anticoagulant functional difference is apparently exclusive to the APTT pathway because such functional differences between hirudin and efegatran were not observed in their respective effects on the PT pathway and no different effects of efegatran and hirudin could be found in the inhibition of other protease clotting factors (109–111). Therefore, the anticoagulant functional selectivity difference found between efegatran (APTT/TT ratio in the range of 30 to 55) and hirudin (APTT/TT ratio about 2 to 3) remains unexplained. The practical result of the mechanistic difference is that upon increasing doses of efegatran in vitro, in animal studies and in clinical use, the TT will become progressively and markedly prolonged without initially affecting the APTT.

In Vivo Studies in Animal Thrombosis Models

Efegatran, given in a constant infusion, was tested in a canine model of coronary artery thrombosis. Efegatran produced dose-depen-

dent anticoagulant effects in the anesthetized dog (112,113). Efegatran at a median dose of 1.0 mg/kg/hr caused a more than 10-fold increase in TT, but only a 1.7-fold increase in APTT. Peak APTT changes were 1.1 ± 0.02 , 1.5 ± 0.07 , 1.6 ± 0.1 , 2.4 ± 0.2 , and 3.3 ± 0.2 fold with 0.25, 0.5, 1.0, 2.0, and 4.0 mg/kg/hr efegatran, respectively. When the infusion of efegatran was stopped, APTT and TT returned to normal within the 2-hour washout period. All doses produced significant prolongation in time to total thrombotic occlusion. The dose-response curve resembled more of a "all or none" profile with 0.5 mg/kg/hr efegatran producing a time to occlusion of 205 ± 2 min compared with 213 ± 14 min for the 4.0 mg/kg/hr efegatran dose group. Template bleeding times were significantly increased only at the high dose of 4.0 mg/kg/hr efegatran. Baseline template bleeding times in efegatran-treated groups were 144 ± 5 , 139 ± 13 , 133 ± 10 , and 126 ± 8 seconds for 0.25, 0.5, 1.0, 2.0, and 4.0 mg/kg/hr, respectively.

In the same dog model of coronary artery thrombosis, the combination therapy with minimum effective doses of efegatran enhanced the antithrombotic efficacy compared with heparin (114).

Efegatran was an effective anticoagulant when used as an adjunct during SK-induced thrombolysis in the anesthetized dog (113). Dose-dependent anticoagulant effect in the presence of SK was observed on TT and APTT. Peak TT increases were 526 ± 66 seconds and 793 ± 85 seconds versus baseline values of 37 ± 1 and 36 ± 1 seconds with 0.25 and 1.0 mg/kg/hr efegatran, respectively. Peak APTT increases were 76 ± 6 and 125 ± 4 seconds

onds versus baseline values of 34 ± 1 and 35 ± 1 seconds with 0.5 and 1.0 mg/kg/hr efegatran, respectively. Time to reperfusion in response to SK-induced thrombolysis was 46 ± 10 minutes, whereas control dogs had no reperfusion. All animals receiving SK with either efegatran or ASA alone, or the combination of efegatran and ASA demonstrated reperfusion of their coronary artery. In the groups receiving SK alone and SK and 0.5 mg/kg/hr efegatran, all vessels that reperused, reoccluded. The time to reocclusion for the SK alone group was 89 ± 23 minutes. Even though all animals receiving 0.5 mg/kg/hr efegatran reoccluded their coronary artery, the time to reocclusion was significantly longer (156 ± 19 min, $p < 0.05$) when compared with the SK alone group. In the group receiving 1.0 mg/kg/hr efegatran, the time to reocclusion was significantly prolonged (198 ± 12 minutes). In the group receiving SK and ASA, the time to reocclusion was 141 ± 32 min, not significantly different from the SK-treated group. The best antireocclusive efficacy was observed in the group receiving SK, 0.5 mg/kg/hr efegatran, and ASA. All vessels exposed to this regimen were patent at the end of the experiment (4 hours).

Thrombolytic therapy with SK in the dog produced a significant increase in template bleeding time (316 ± 50 sec versus 140 ± 10 sec, $p < 0.05$) (114). Anticoagulant and/or antiplatelet therapy with efegatran and ASA had no significant additive effect on template bleeding time beyond that induced by SK alone.

Studies in Human Volunteers

With a 15-minute i.v. infusion of efegatran in human volunteers, a dose of approximately 0.025 mg/kg was required to double the TT value. TT was a specific and extremely sensitive measure of the anticoagulant activity of efegatran. The upper limit of quantification for TT (120 seconds for the automated method chosen) was exceeded at doses above 0.1 mg/kg, making interpretation of the anti-

coagulant effect of efegatran, in terms of TT prolongation, more difficult. At higher dose levels of efegatran (0.225 mg/kg to 0.3 mg/kg), APTT values were prolonged in a dose-dependent fashion. Immediately before the termination of the 15-minute efegatran infusion of 0.3 mg/kg, APTT prolongation ranged from 175% to 238% of baseline. The offset of anticoagulant effect (measured by prolongation of APTT) after cessation of drug infusion was rapid, with a pharmacologic half-life for efegatran of approximately 30 minutes.

Prolonged i.v. administration of efegatran (0.2, 0.4, 0.6, and 0.8 mg/kg/hr) produced dose-related anticoagulant activity with no accumulation of effect. For all infusion rates, except 0.2 mg/kg/hr, the TT was prolonged to greater than the 120-second limit of the automated technique used; however, dose-related prolongations of APTT were observed. The anticoagulant effect of efegatran, measured by APTT prolongation as a percentage of baseline values, correlated in a linear fashion ($p < 0.001$) with the rate of infusion of efegatran, irrespective of the duration of infusion.

The template bleeding time was used in healthy male subjects as a surrogate assessment of bleeding risk. Bleeding times fell within the reference range for the method (2 to 10 minutes), irrespective of the dose level or duration of infusion of efegatran studied. In a few individuals, sporadic prolonged bleeding time measurements were recorded, without clear evidence of a dose-response relationship to efegatran. Prolonged bleeding times had returned to baseline by the time a follow-up assessment was made 6 hours post-termination of infusion.

Clinical Studies

The preclinical and clinical pharmacology of efegatran was recently reviewed (115).

Safety and anticoagulant properties of efegatran were studied at three dose levels in 36 patients with unstable angina (116). Three groups of 10 patients have been treated with a

loading dose of 0.1 mg/kg in combination with a 48-hour infusion of 0.10, 0.32, and 0.63 mg/kg/hr, respectively. Six patients were randomly allocated to receive APTT-adjusted unfractionated heparin (5,000-IU bolus followed by 1,000 IU/hr). In contrast to treatments with heparin, no APTT overshoot at 0.5 hour was apparent.

At these dose levels, efegatran has been clinically well tolerated. One patient at the highest dose received 2 U packed cells for a hematoma after cardiac catheterization. Recurrent ischemia during infusion occurred in four, one, and four patients receiving efegatran at the respective dosages indicated above, as well as in three patients receiving heparin. No clinically significant prolongations of bleeding time have been recorded in any of the patients treated through dosages of 0.84 mg/kg/hr. The estimated clinically effective dosage is 0.63 mg/kg/hr.

APTT measurements show that efegatran produces a dose-dependent prolongation of APTT as predicted from both preclinical and human volunteer data. The APTT effect correlates linearly with efegatran plasma concentration. The i.v. half-life of APTT effect and efegatran plasma concentration was 35 minutes, and clearance from plasma was rapid (0.4 L/hr/kg). Eighty-five percent of the steady-state concentrations were achieved 2 hours after starting a constant rate infusion. There is no evidence of accumulation of anticoagulant effect over time.

Whether efegatran given for 72 to 96 hours and adjusted to APTT can reduce the patency lag associated with SK was studied in 247 patients with acute myocardial infarction in the randomized, open-label, dose-finding ESCALAT study (117). The combination of efegatran and SK was compared to t-PA and i.v. heparin using angiographic and clinical endpoints. The study has been completed but not reported. In a second dose ranging trial, heparin or escalating dosages of efegatran (0.3 to 1.2 mg/kg/hr) were compared in 330 patients with acute myocardial infarction (118). This trial was also completed but not

reported in detail. Further clinical trials with efegatran are not being pursued.

NAPSAGATRAN

Napsagatran (RO 46-6240) is a cyclopropyl derivative of a novel class of thrombin inhibitors bearing a 3-(aminomethyl)-amidinopiperidine as an arginine side chain mimetic. An attached L-aspartic acid serves as template to reach two hydrophobic pockets near the active site of the enzyme (119).

Napsagatran is a selective, potent, competitive, and reversible inhibitor of thrombin with low molecular weight (559 daltons). It inhibits the catalytic activity of thrombin toward fibrinogen or the chromogenic substrate 2238 at picomolar concentrations. In the tests the compound is approximately two orders of magnitude more potent than argatroban. This activity is also evident in clotting tests performed in human plasma such as PT, TT, PT, and APTT (120).

Many enzymes of the coagulation system are closely related to thrombin. Napsagatran represents one of the most specific synthetic thrombin inhibitors of small molecular weight known today. Specificity for thrombin is an important parameter for an antithrombotic compound because interference with most of the related enzymes is undesirable. To estimate the selectivity of napsagatran, inhibition of several serine proteases from different physiologic systems was determined. The selectivity ratio of napsagatran for trypsin was indicated by the ratio K_i for trypsin/ K_i for thrombin and amounted to 7140 (119-121). The selectivity ratios for plasmin, t-PA, kallikrein, C1-esterase, elastase and chymotrypsin range from 8,600 to 250,000.

Napsagatran inhibits clot-bound thrombin and thrombin in solution with equal potency whereas hirudin is less active against clot-bound than against fluid-phase thrombin (122). It remains to be seen whether this interesting experimental property of napsagatran will translate into an increased therapeutic

tic benefit in clinical situations of a preexisting clot.

Napsagatran efficiently inhibits fibrin deposition on tissue factor expressed by human endothelial cells. The procoagulant activity of tumor necrosis factor- α -stimulated monolayers of human endothelial cells was studied in a flow system with human venous blood using desirudin, napsagatran, and heparin. Under venous blood flow conditions (at wall shear rates of 65 seconds⁻¹), these compounds inhibited fibrin deposition by 50% at concentrations of 14, 28, and 412 ng/ml, respectively (123).

The shear rate-dependent and perfusion time-dependent effect of napsagatran at 100 $\mu\text{g/kg/min}$ on thrombogenesis induced by subendothelium of rabbit aorta were studied using an ex vivo perfusion chamber system (124). The deposition of fibrin on subendothelium was completely abolished at shear rates of 100, 650, and 2,600 seconds⁻¹ after 5- and 30-minute perfusions. In contrast, a significant effect on thrombus formation after a 5-minute perfusion could be observed only at a shear rate of 100 seconds⁻¹, whereas after a 30-minute perfusion thrombus formation was reduced at all three shear rates. These results show that thrombin-mediated mechanisms are important in the latter phase of thrombus growth in this thrombosis model.

In Vivo Experimental Thrombosis Models

Napsagatran was compared with heparin in a canine model of coronary thrombosis (121). Occlusive thrombosis of the left circumflex coronary artery was induced by electrical injury. In parallel, arterial subendothelium was exposed to native blood using an annular perfusion chamber for 5, 10, and 20 minutes at a wall shear rate of 650/sec. Dogs received saline, heparin (40 and 70 IU/kg/hr), or napsagatran (3 and 10 $\mu\text{g/kg/min}$). Heparin (40 IU/kg/hr) and napsagatran (3 $\mu\text{g/kg/min}$) delayed or prevented in vivo thrombotic occlusion, but only napsagatran (10 $\mu\text{g/kg/min}$) significantly decreased the intracoronary

thrombus when compared with saline. High-dose unfractionated heparin (70 IU/kg/hr) or napsagatran (10 $\mu\text{g/kg/min}$) decreased the platelet-rich thrombus after a 20-minute chamber perfusion. Neither heparin nor napsagatran decreased the thrombus volume after a 5-minute perfusion. Heparin (70 IU/kg/hr) and napsagatran (10 $\mu\text{g/kg/min}$) prolonged the APTT differently (more than six-fold and 1.4-fold, respectively, $p < 0.01$), whereas the ACT was prolonged equally (2.5-fold). Thus, napsagatran in this dog model shows arterial antithrombotic effects similar to those of heparin.

In a guinea pig model of arterial thrombosis, cyclic variations of blood flow (CFV) in the carotid artery were monitored after mechanical damage of the vessel. These CFVs indicated build-up and embolization of platelet-rich thrombotic masses. Napsagatran, desirudin, and heparin applied as an i.v. bolus followed by a continuous i.v. infusion prevented the occurrence of CFVs in a dose-related manner (120). Thus, at a dosage of 30, 50, and 67 $\mu\text{g/kg/min}$, respectively, the compounds significantly inhibited the occurrence of CFVs without prolonging the bleeding time.

The antithrombotic effect of napsagatran was also evaluated in an acute in vivo venous stasis thrombosis model (Wessler model) in the rat (121). Napsagatran completely inhibited the appearance of a clot at a dosage of 3 $\mu\text{g/kg/min}$ (ID_{100}). A partial inhibition was observed at 1 $\mu\text{g/kg/min}$. At these dosages the APTT was prolonged 1.6- to 2.5-fold in comparison with controls. As expected, recombinant hirudin was also fully effective and overall about three times as potent as napsagatran. In contrast, as compared with napsagatran, about five times more heparin was needed for a full inhibition ($\text{ID}_{100} = 20 \mu\text{g/kg/min}$) and this dose of heparin prolonged the APTT 12-fold.

Clinical Studies

Napsagatran is currently in phase II clinical trials to establish its efficacy and safety for

preventing postoperative thrombosis as well as treating established venous thrombosis.

INOATRAN

Inogatran (H314/27) is a synthetic dipeptide with a molecular weight of 439 daltons (125). Inogatran selectively, rapidly, and competitively binds thrombin. In vitro it doubles the plasma TT at a concentration of 23 nmol/L and the APTT at 1.1 μ mol/L. Thrombin-induced platelet aggregation is inhibited at an IC_{50} of 17 nmol/L.

Inogatran was evaluated in three rat models of thrombosis. In the venous thrombosis model, inogatran dose-dependently inhibited thrombus formation with a more than 80% antithrombotic effect at a plasma concentration of 0.45 μ mol/L-1 (126). In an arterial thrombosis model, inogatran dose-dependently inhibited thrombus formation and preserved vessel patency and the mean blood flow. Acetylsalicylic acid potentiated the effects of low plasma concentrations of inogatran in the arterial thrombosis model. In a model of rt-PA-induced thrombolysis of a thrombus in the carotid artery, the patency time and the cumulative blood flow improved more in the presence of inogatran during the 2-hour thrombolysis period than with rt-PA alone. At a high therapeutic plasma concentration of inogatran, there was only a moderate prolongation of bleeding time compared with the control value. A study was designed to examine the modulation of coronary artery reocclusion by inogatran with or without aspirin (127). Twenty-two dogs with electrically induced occlusive intracoronary thrombus were treated with saline or different doses of inogatran (up to 0.25 mg/kg bolus followed by 0.6 mg/kg/hr for 2 hours). rt-PA was infused for 20 minutes starting 2 minutes after the bolus in all dogs. Coronary artery blood flow was monitored for 120 minutes after rt-PA administration. Reperfusion rates were similar in all groups, but the time to reperfusion was the longest and the reocclusion rate the lowest in the high-dose inogatran group. Aspirin did not potentiate the effect of suboptimal doses of inogatran.

Studies in Healthy Male Volunteers

Inogatran was studied in healthy male human volunteers with regard to tolerability, pharmacokinetics, and effects on hemostasis. It was given i.v. as a bolus in doses up to 0.4 mg/kg body weight. The highest peak plasma concentration observed was 7 μ mol/L, corresponding to an APTT prolongation of three times. The drug was also given as a constant i.v. infusion over 4 hours at a dosage of 0.3 mg/kg/hr, which resulted in a mean plasma concentration at steady state of 1.9 μ mol/L and an APTT prolongation of 2.3 times. The drug was well tolerated and without side effects with the exception of a slightly increased bleeding tendency at the blood sampling site. Inogatran had a volume of distribution of 0.2 ml/kg and a total plasma clearance of 6 ml/min/kg, resulting in a terminal half-life of about 1 hour. The drug was not metabolized and it was excreted unchanged with the elimination evenly distributed between urine and feces. Ex vivo the TT was linearly correlated to the plasma concentration while the APTT concentration curve was nonlinear. At the highest plasma concentrations a slight prolongation of the capillary bleeding time was seen in some subjects. Markers of thrombin activity (thrombin-antithrombin complex and prothrombin F1+2) decreased during the constant infusion of the drug. There was no effect on fibrinolysis (PAI-1 and t-PA activities) or protein C.

Clinical Development

In an open-design study, 37 patients with unstable angina or non-Q-wave infarction were treated within 72 hours of symptoms with aspirin and other standard treatment and were allocated consecutively to groups receiving a 4-hour infusion with one of three doses of inogatran (bolus of 0.035, 0.07, and 0.1 mg/kg followed by a 4-hour infusion of 0.035, 0.126, and 0.189 mg/kg/hr) (128). There was a predictable dose-response relationship between inogatran concentration and APTT. Thrombin generation (prothrombin F1-

was suppressed after 4 hours of treatment compared with baseline, but thrombin activity (fibrinopeptide A) was not suppressed. There were no adverse hemodynamic or other effects. Minor bleeding was noted in 37% of the patients. During the first 4 hours after inogatran treatment, thrombin activity and episodes of ischemia were increased compared with the inogatran infusion period.

Three doses of inogatran were studied in 1,209 patients with unstable angina or non-Q-wave myocardial infarction in a randomized double-blind study (129). After a bolus of inogatran (1.10, 2.75, or 5.50 mg/kg) or heparin, infusions continued for 3 days with a low (2.0 mg/kg/hr), medium (5.0 mg/kg/hr), or high (10.0 mg/kg/hr) dosage of inogatran (resulting in APTT prolongations of 1.3, 1.5, and 1.8 times baseline at 24 hours, respectively) or heparin (1,200 IU/hr). A composite endpoint of death, myocardial infarction, or refractory or recurrent angina at 3, 7, and 30 days did not show differences between the groups. Thus, inogatran at the doses used was no better than heparin in preventing ischemic coronary events. There was no dose-effect relationship concerning inogatran. After cessation of infusion, hypercoagulability was noted in both the heparin and the inogatran groups.

Further clinical development of inogatran has been stopped.

OTHER DIRECT ANTITHROMBINS

A variety of other antithrombins have been synthesized but no clinical trials have been planned. Among them the peptide PPACK (d-Phe-Pro-Arg-CH₂Cl) (RWJ-27755) was found to be a highly potent irreversible inhibitor of thrombin by alkylating the active center site histidine (130–132). There is a rapid loss of activity of this compound due to reactions with other plasma components (133). Notwithstanding its antithrombotic effect at high doses in different arterial thrombosis models in the rat, dog, and pig, its alkylating properties have considerably tempered the enthusiasm for its clinical development. Boroarginine derivatives (DuP714) are potent

antithrombins with a potential for oral bioavailability (134). Unfortunately, their boron constituent induces liver toxicity. CVS-1123 (or CH₃CH₂CH₂)₂-CH-CO-Asp(OCH₃)-Pro-Arg-CHO) is a synthetic peptidometric of small molecular weight (575 daltons). It is a slow, competitive inhibitor of the amidolytic activity of thrombin as well as a potent anticoagulant in plasma *in vitro*. CVS-1123 has been shown in an anesthetized porcine model to be effective in preventing arterial thrombus formation (135). The oral antithrombotic efficacy of this compound, administered for 24 hours, has been demonstrated in the conscious canine dog to prevent primary thrombus formation after deep arterial coronary wall injury (136).

Other antithrombins still in development are hirudisin–hirudin derivatives combining blocking of the platelet IIb/IIIa receptor and direct thrombin inhibition. In the hirudisins, residues 32 to 35 of hirudin have been replaced by the integrin motif RGDS and KGDS, obtaining a potent thrombin inhibitor (K_D 0.16 to 0.26×10^{-12} mol/L compared with 0.2×10^{-12} mol/L for desirudin) with additional disintegrin activity (137). In addition to inhibiting GPIIb/IIIa receptor-dependent platelet interactions, the platelet-binding integrin motif is expected to target the antithrombin action of hirudin to platelets, possibly allowing lower and safer doses of desirudin in the treatment of thrombotic disease (138). Similarly, desirudin targeted to fibrin by coupling them to Fab' portions of antibodies directed against platelet GPIIb/IIIa receptor or fibrin β -chain (139,140) may allow for highly efficient antithrombosis at doses lower than presently required for desirudin.

Aptamers are oligonucleotides (double- or single-stranded DNA or single-stranded RNA) some of which bind directly to thrombin with binding affinities in the range of 20 to 200 nmol/L (e.g., a single-stranded, 15-nucleotide DNA aptamer) (141). This potent aptamer with rapid onset of action and short half-life has been found to interact with the anion-binding exosite of thrombin, so that it competes with substrates that interact with

that specific site, such as fibrinogen and thrombin platelet receptors (142). This aptamer has been shown to reduce arterial platelet thrombus formation in an animal model, as well as to inhibit clot-bound thrombin in an *in vitro* system (143).

Recently, there has been a report of a novel synthetic thrombin inhibitor, CVS #995, comprised of 19 amino acids, in which recognition sequences for the catalytic and primary exosite binding domains of thrombin have been linked by a transition state analogue (144). Catalytic inhibition of thrombin is obtained in the picomolar range for this slow and tightly binding thrombin inhibitor. When compared with bivalirudin, this agent was superior at inhibiting platelet aggregation and venous thrombosis in a rat model.

A number of lysyl α -ketocarbonyl derivatives were found to be as potent (on a weight basis) as hirudin when evaluated in a rat arterial thrombosis model (145). Their modest oral bioavailability (10% to 19%) suggests the possibility that α -keto amide containing thrombin inhibitors may have utility as orally active antithrombotic agents.

The main platelet receptor for thrombin (Fig. 9-5) was recently characterized (147) and has the unique feature of a tethered ligand. After thrombin binds the receptor through a hirudinlike anion-binding exosite, it cleaves the receptor to show a new receptor's amino acid terminus, which functions as the tethered ligand to activate the receptor. The molecule of thrombin thus remains free to activate other receptor sites and propagate the thrombotic process (148). This receptor has been cloned (149). A polyclonal antibody was raised against the peptide derived from the thrombin-binding exosite region of the cloned human thrombin receptor (150). This antibody serves as a selective inhibitor of the thrombin receptor.

CONCLUSION

1. Despite interdigitation of residues critical to its opposing biologic functions, the pro- and anticoagulant properties of

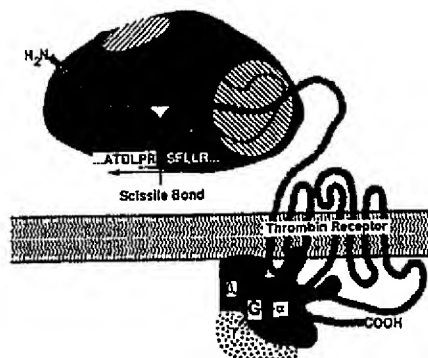


FIG. 9-5. Mechanism of thrombin receptor activation. Cleavage by thrombin of the Arg⁴¹-Ser scissile bond in the native thrombin receptor shows the new amino terminal Ser-Phe-Leu-Arg ... sequence of the receptor, which serves as a tethered agonist ligand that directly activates the thrombin receptor, resulting in postreceptor signaling via specific G proteins. (From Ogletree, ref. 146, with permission.)

thrombin have been related to fast and slow allosteric forms of the enzyme, respectively, corresponding to distinct conformational states of the protein. None of the site-directed thrombin inhibitors blocks the procoagulant function only.

2. Advantages of all site-directed thrombin inhibitors over heparin are (a) selectivity and rapidity for interacting with functional domains of thrombin; (b) ability to inhibit both clot-bound thrombin and thrombin in the fluid phase; (c) independence of plasma proteins for their activity; (d) absence of neutralization by natural anticoagulants, plasma proteins, endotoxin and platelet factor 4; (e) predictable pharmacokinetic and pharmacodynamic profile; and (f) absence of immunologic reactions. For none of the specific site-directed thrombin inhibitors is an antidote available, and it has proven difficult to synthesize orally available specific thrombin inhibitors.
3. Desirudin at the low doses used in large scale clinical trials in patients with a myocardial infarction treated with

- teplase or SK appears to be at least as effective as unfractionated heparin in terms of therapeutic benefit. Its therapeutic window in this setting is narrow because significantly more moderate bleeding occurs during i.v. desirudin than during unfractionated heparin administration.
4. In patients with unstable angina, low- and medium-high doses of desirudin resulted in a trend toward lower event rates at 7 days compared with unfractionated heparin at the cost of significantly more minor bleeding.
 5. All trials to date have studied relatively short-term (3 to 5 days) desirudin administration. The potential benefits (or lack thereof) of longer-term administration of desirudin in unstable coronary syndromes and PTCA remain unknown.
 6. In the prevention of postoperative deep venous thrombosis after orthopedic surgery, desirudin is significantly more effective than unfractionated heparin and low molecular-weight heparin without increase in bleeding risk.
 7. There is a large clinical experience with bivalirudin in the treatment of unstable angina and as adjunctive therapy with thrombolytic drugs for acute myocardial infarction. Bivalirudin appears to be as effective as unfractionated heparin in the prevention of acute complications of PTCA in patients with unstable angina, but is significantly more effective than heparin in the cohort of patients with postinfarction angina. This advantage is associated with a 60% reduction of major bleeding complications. However, there was no significant benefit on event-free survival at 7 months in the subgroup.
 8. Argatroban is more effective than heparin in animal models of thrombosis, but it is not demonstrated whether this is the case in patients. A drawback of the reversible thrombin inhibitor argatroban is that an infusion of short treatment may induce a thrombotic rebound phenomenon after cessation of infusion in clinical conditions in which the tendency toward thrombosis persists for days.
 9. The safety and efficacy of hirunorm, efegatran, and napsagatran remain to be established through ongoing clinical trials. The clinical experience with inogatran has engendered dismay because no superiority over unfractionated heparin could be demonstrated in a large trial in patients with unstable angina or non-Q-wave infarction.
 10. Several of the direct thrombin inhibitors also exhibit non-thrombin-mediated actions that are responsible for some of the additional pharmacologic effects that may also contribute to the antithrombotic/hemorrhagic balance. Direct thrombin inhibitors should be considered as a class of drugs, each compound having its own pharmacologic and therapeutic profile.
 11. Direct thrombin inhibitors are easy to use, having a stable anticoagulant activity, lack of direct effects on platelet function, and uniformity of pharmacologic composition and activity, but no antidote is available.

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Specific Factor Xa Inhibitors

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Today factor Xa targeting is one of the major focuses of drug development. The factor Xa inhibitor strategy was actually derived, in part, from heparin. The low molecular weight (LMW) heparins have higher anti-Xa activity than antithrombin (AT) activity, whereas heparin has equal factor Xa and thrombin inhibitory activities. An indirect evidence of the validity of the hypothesis that factor Xa inhibition is important for the control of thrombogenesis is given by the clinical antithrombotic efficacy of LMW heparins, which contain a large proportion of molecules with high anti-factor Xa activity (1). Pharmacologic development has now separated these two properties so that solely anti-factor Xa or AT agents are available.

The first development of factor Xa inhibitors was met with less interest than throm-

bin inhibitors. These early inhibitors had low affinity to factor Xa, low selectivity, and low potency (2,3). Because of the increase in enzymatic activity of the coagulation cascade once the prothrombinase complex is formed, a potent factor Xa inhibitor is required to have an extremely high affinity for the enzyme. The first factor Xa inhibitors did not fulfill this requirement.

Potent factor Xa inhibitors have several potential advantages. Factor Xa is in the common pathway of both the intrinsic and extrinsic systems, playing a central role in the coagulation pathway, so it is a logical focus of drug development for the control of thrombosis. Factor Xa is formed at an earlier stage than thrombin, and the procoagulant effect of factor Xa is strongly amplified by the prothrombinase complex. Factor Xa has no

known activity other than as a procoagulant, as opposed to thrombin, which has multiple activation roles at various plasmatic and cellular levels (platelets, endothelial cells, smooth muscle cells, other cells), not the least of which is activation of the protein C inhibitor pathway. Inhibition of protein C by a thrombin inhibitor would lead to a reduced formation of activated protein C, an important natural anticoagulant.

Because factor Xa has relatively slow activation kinetics, as opposed to thrombin, opposing its function should result in easier management of the balance between the therapeutic and bleeding effects of a drug. From recent clinical trials, specific thrombin inhibitors have shown to have a relatively narrow safety/efficacy margin that could lead to an overdose of the therapeutic dose with a resultant bleeding complication (4,5). Because of their different mechanism of action, factor Xa inhibitors are expected to have a better efficacy/safety profile than thrombin inhibitors.

CLASSIFICATION

Factor Xa inhibitors are structurally diverse, ranging from peptides to proteins to heparin saccharidic sequences (6,7). They can be either naturally derived, recombinant, or synthetic in origin. Molecular size differs between the inhibitors, as does specificity and kinetics of factor Xa inhibition. The targeted binding site on factor Xa can differ between

the inhibitors; they can be direct binding to factor Xa or indirect via a cofactor such as AT III. Binding to the enzyme can be either reversible or irreversible. The protein inhibitors are limited in the degree of activity they produce. Two possible reasons for this limitation are (a) size that limits access of the inhibitor to factor Xa bound within clots, and (b) when thrombin is bound to fibrin, the heparin binding site on thrombin is inaccessible for heparin. Other mechanisms may exist. Furthermore, protein inhibitors tend to be immunogenic, can carry viral or animal contaminants, and can become limited in supply.

Because of the structural differences, the primary mechanism of action (i.e., direct or indirect acting on factor Xa as well as other attributes) differs with the various agents. The factor Xa inhibitors that are in development are shown in Table 10-1. Only a few of the agents have moved out of the experimental and into the clinical realm.

MECHANISMS OF ACTION

Coagulation

The inhibition of factor Xa has multiple consequences for the plasmatic coagulation system (Fig. 10-1) as well as for cellular reactions that result in an effective anticoagulation and the prevention of thrombotic processes. Based on the amplification mechanisms in the coagulation cascade and the important role of the prothrombinase complex, highly effective

TABLE 10-1. Factor Xa inhibitors

Agent	Chemical nature	Source	Developmental status
Direct Inhibitors			
Yagin	Medicinal leech protein (85 amino acids)	Animal derived	Terminated
Antistasin	Mexican leech protein (119 amino acids)	Recombinant	Terminated
TAP	Tick protein (80 amino acids)	Recombinant	Preclinical
NAP-5	Hookworm protein	Recombinant	Preclinical
TFPI	Human protein	Recombinant	Preclinical
DX-9065a	Propanoic acid derivative	Synthetic	Phase II clinical trial
SEL 2711	Pentapeptide produced by combinatorial chemistry	Synthetic	Preclinical
YM-60828			Preclinical
Indirect inhibitors			
Heparin	Oligosaccharide; requires binding to AT III	Synthetic	Phase II clinical trial
pentasaccharide			

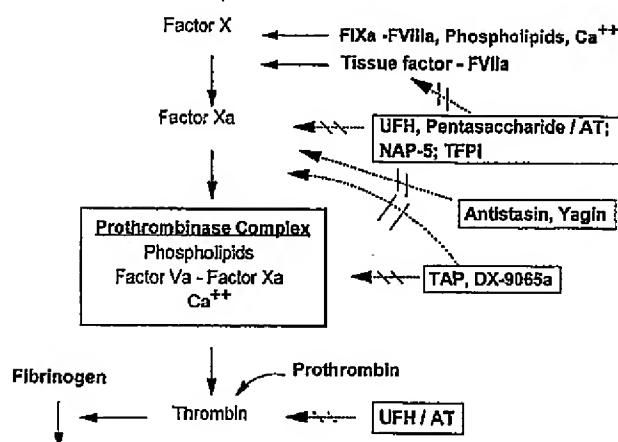


FIG. 10-1. Schematic representation of the coagulation cascade and the sites of action of factor Xa inhibitors. UFH, unfractionated heparin; AT, antithrombin III; —, inhibition.

and selective factor Xa inhibitors are expected to strongly inhibit the generation of thrombin. This is probably the most important mechanism of factor Xa inhibitors with regard to their antithrombotic effectiveness. By this action, thrombin mediated positive feedback reactions such as the activation of the cofactors V and VIII that amplify thrombin formation, and the effect of thrombin on platelets and other cellular elements are also altered.

Free Versus Bound Factor Xa

An important aspect of the mechanism of the antithrombotic effect of factor Xa inhibitors is their capacity to inhibit clot-bound factor Xa. Because of the small size (500 to 800 daltons) of some of these inhibitors, they are able to inhibit bound prothrombinase as well as free factor Xa. Tick anticoagulant peptide (TAP) is particularly capable of inhibiting bound factor Xa.

Remnants of intravascular thrombi, known to induce activation of the coagulation system, may play a role in rethrombosis and restenosis after coronary thrombolysis. Pre-existing thrombin, which is bound to fibrin and re-exposed during thrombolysis, has been thought to be the primary mediator of thrombus-associated procoagulant activity; thus,

thrombin inhibitors such as hirudin may be useful agents to prevent the recurrence of thrombosis after thrombolysis (8). However, recent results indicate that other clotting factors such as factor Xa are also bound to whole blood clots. Therefore, activation of prothrombin by clot-associated factors Xa/Va can significantly contribute to the procoagulant activity of intravascular thrombi (9).

The activity of clot-bound factor Xa is resistant to inhibition by AT III and AT III-dependent inhibitors such as the heparin pentasaccharide (due to its size or other mechanisms) (9,10). On the other hand, the activation of prothrombin can be inhibited by direct acting factor Xa inhibitors such as TAP as well as by tissue factor pathway inhibitor (TFPI) (10,11).

Restenosis

The inhibition of the proliferation of vascular smooth muscle cells (VSMCs) also may be controlled by factor Xa. Migration and proliferation of VSMCs as a reaction to injury of the endothelium and the resulting formation of a neointima mainly contribute to the development of restenosis and atherosclerosis. Platelets, thrombin, and other components of the thrombotic process are important factors in neointimal formation (12-15).

The serine protease thrombin is known to exert, besides its action in the plasmatic coagulation system, several cellular effects via the reaction with its specific receptor. By this mechanism it activates platelets and acts as a strong mitogen for endothelial cells, VSMC, fibroblasts, and macrophages. In limited studies on mitogenesis in cultured rat VSMCs, it was shown that factor Xa is also a potent mitogen that stimulates DNA synthesis and cell growth in VSMCs (16,17).

Most probably factor Xa exerts its effect indirectly via the platelet-derived growth factor (PDGF) receptor tyrosine kinase pathway. Factor Xa stimulates VSMCs to release pre-existing PDGF, which then, through the receptor tyrosine kinase pathway, leads to the activation of mitogen-activated protein kinases (MAPK) which are well-characterized intracellular mediators of cell proliferation (16). This action of factor Xa on VSMC seems to be related to its serine protease activity because in the presence of specific factor Xa inhibitors such as antistasin and TAP the mitogenic effect of factor Xa is blocked (16,17).

VSMCs proliferation, which is mediated by factor Xa, also might play an important role in reocclusion and restenosis after angioplasty in vivo. Therefore, specific inhibition of factor Xa can be expected to limit intimal hyperplasia after damage of the vascular endothelium and, thus, to diminish the restenosis rate after successful angioplasty.

Under experimental conditions the specific factor Xa inhibitors antistasin and TAP have been shown to limit restenosis after balloon angioplasty (18,19). A 2-hour infusion of antistasin resulted in significantly less restenosis and less luminal narrowing by plaque measured 28 days after balloon angioplasty of atherosclerotic femoral arteries in rabbits compared with controls (18). In a porcine model of severe coronary artery injury, a short-term administration of TAP for 60 hours resulted in a long-term decrease in neointimal thickness measured 28 days after injury (19).

These results implicate specific factor Xa inhibitors as effective substances to reduce neointimal hyperplasia either by preventing

the mitogenic effects of factor Xa and/or by inhibiting the generation of thrombin which by itself is also a potent mitogen.

DIRECT INHIBITORS

Antistasin

Antistasin, purified several years ago from the Mexican leech (*Haementeria officinalis*), has an apparent molecular weight of 17,000 daltons (20). Due to antibody formation, it has fallen out of developmental interest and is no longer being developed. It inhibits factor Xa by forming a stable enzyme-inhibitor complex (21). Antistasin was more effective at prolonging the prothrombin time (PT) than hirudin, but only slightly less effective than hirudin at prolonging the activated partial thromboplastin time (APTT) (6).

Yagin

Yagin, an 85-amino acid peptide isolated from the medicinal leech (*Hirudo medicinalis*), has 50% homology with antistasin. It is a slow, tightly binding inhibitor of factor Xa, where the inhibition is a time-dependent reaction effected by the order of addition of components (22). Optimization of product methods is in progress.

Tick Anticoagulant Peptide (TAP)

TAP was originally isolated from the tick *Ornithodoros moubata*. It is now produced through recombinant technology. It is a 60-amino acid peptide (6,850 daltons) and is a slow, tightly binding inhibitor of human factor Xa that inhibits the enzyme via a two-step mechanism. Initially it forms a relatively weak complex with factor Xa, followed by a more stable enzyme-inhibitor complex (23,24). TAP is characterized by a much higher affinity of the inhibitor to the enzyme when it is assembled in the prothrombin complex with an appropriately lower inhibition constant (K_i value 0.006 nmol/L versus 0.18 nmol/L for free factor Xa) (25,26).

Like other naturally derived inhibitors, TAP effectively inhibits factor Xa and has shown antithrombotic effects in various thrombosis models. Using TAP as a very selective and highly effective factor Xa inhibitor, it was shown under experimental conditions that the inhibition of thrombin generation is an effective approach to affect processes of thrombosis and restenosis. TAP inhibited *in vivo* the formation of venous thrombosis (27–29) and prevented platelet and fibrin deposition as well as thrombus formation in arterial thrombosis and in arteriovenous shunts (29–31) as well as in *ex vivo* human non-anticoagulated blood (32). It also accelerated perfusion during thrombolysis and prevented acute reocclusion in canines (33–35).

TAP has a different anticoagulant profile as measured by the APTT (26,27). In comparison with antistasin, for example, both *in vitro* and *ex vivo*, TAP was found to be much less potent than antistasin in prolonging the APTT despite nearly equal antithrombotic efficacies in a rabbit model of venous thrombosis. This reflects kinetic differences in the rate of factor Xa inactivation between various inhibitors, i.e., TAP shows a time dependent inhibition requiring an incubation period of 50 to 60 minutes to achieve maximal inhibition (27). On the other hand, in the celite activated clotting time (ACT) assay, TAP showed the most potent anticoagulant activity in comparison with heparin and other factor Xa inhibitors (Fig. 10-2). These differences show that the antithrombotic efficacy of a given anticoagulant cannot always be predicted by clotting assay values.

Besides the antithrombotic effectiveness of TAP found in experimental venous and arterial thrombosis, this factor Xa inhibitor showed favorable actions against the restenosis processes. TAP reduced angiographic restenosis and caused less luminal cross-sectional narrowing by plaque after short-term administration to rabbits (18), led to a long-term decrease in neointimal thickness in damaged pig coronary arteries (12), and inhibited mitogenesis in cultured rat aortic smooth muscle cells (17).

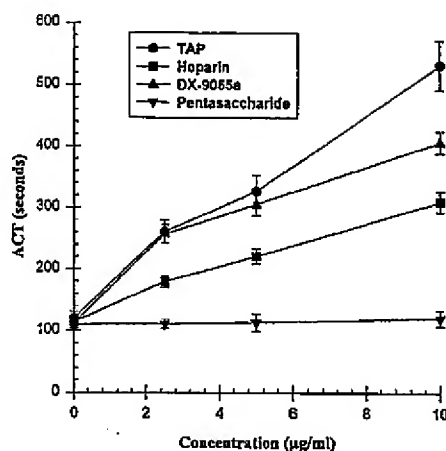


FIG. 10-2. In a comparative study the anticoagulant effects of TAP, DX-9065a, and heparin showed concentration-dependent effects in a celite-activated whole-blood clotting assay (ACT). At equigravimetric concentrations, TAP produced the strongest effect, followed by DX-9065a, and a weaker effect by heparin. Pentasaccharide, on the other hand, did not produce significant prolongation of the time to clot. These results demonstrate that in a test system where thrombin is generated by a strong activator, certain factor Xa inhibitors can produce a concentration-dependent anticoagulant effect whereas other factor Xa inhibitors do not have an effect, depending on their mechanism of action.

Factor Xa and the *de novo* activation of prothrombin may play an important role in the procoagulant activity of intravascular thrombi that may lead to reoccurrence of thrombosis after thrombolysis and to propagation of thrombi (9,36). The clot-associated factor Xa activity can be inhibited by TAP but not by AT III and AT III-dependent inhibitors (9,36).

NAP-5

NAP-5 is one of a family of anticoagulant proteins isolated from hookworm nematodes. It has a molecular weight of 8,700 daltons and inhibits factor Xa and the factor VII/TF complex after prior binding to factor Xa (37). It is in preclinical trials.

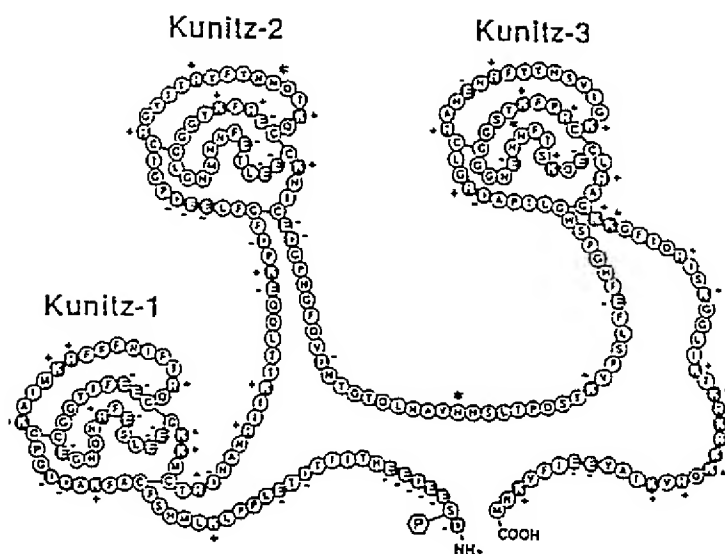


FIG. 10-3. Structure of tissue factor pathway inhibitor (TFPI), the endogenous inhibitor of the extrinsic coagulation pathway. (From Girard et al., ref. 90, with permission.)

Tissue Factor Pathway Inhibitor

TFPI is a human protein (43 kDa) (Fig. 10-3) identified over 40 years ago (38,39). TFPI is the endogenous inhibitor of the extrinsic coagulation pathway that, in addition to the inhibition of the factor VIIa/tissue factor complex in a final step, also binds to and inhibits factor Xa (9,36). Due to its mechanism of action, TFPI is an important regulator of coagulation. TFPI has been shown to inhibit venous thrombosis (40), rethrombosis after t-PA use (40), and platelet thrombosis after balloon injury to the vessel in experimental animals (41). Heparin and LMW heparins release endogenous TFPI upon administration; none of the factor Xa inhibitors tested to date are associated with the release of TFPI (11). A recombinant form is currently in preclinical trials. Defibrotide also releases TFPI (42).

DX-9065a

The Daiichi compound DX-9065a is a novel type of synthetic factor Xa inhibitor (Fig. 10-4 and Table 10-2). This nonpeptide,

propanoic acid derivative, LMW compound (571 daltons) has been described as a potently acting inhibitor of factor Xa. It inhibits factor Xa in a competitive manner with an inhibition constant in the nanomolar range (43,44). DX-9065a is also highly selective for factor Xa and shows limited oral absorption (44-47). It is in phase II clinical trials.

The factor Xa inhibitor DX-9065a is a promising antithrombotic agent. Effective antithrombotic activity was found in models of venous (Fig. 10-5) and arterial thrombosis (29,40,43,48,49), arteriovenous shunt thrombosis (29,43,50,51), acute disseminated intravascular coagulation (43,51-53) as well as hemodialysis in cynomolgus monkeys (

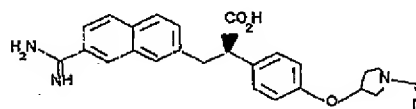


FIG. 10-4. Structure of DX-9065a, the synthetic nonpeptide factor Xa inhibitor. (From Dr. S. Iwata, with permission.)

TABLE 10-2. Characteristics of DX-9065a and the heparin pentasaccharide

Pentasaccharide	DX-9065a
Synthetic	Synthetic
Oligosaccharide	Propanoic acid derivative
1,728 daltons	571 daltons
AT III-pentasaccharide complex binds factor Xa	Direct binding to factor Xa
Limited inhibition of clot-bound/prothrombinase-bound FXa	Inhibits clot-bound/prothrombinase-bound factor Xa
Prolongs the Heptest	Prolongs the APTT and PT
Does not prolong the APTT or PT	Does not prolong the Heptest
No platelet interactions in normal and HIT systems	No platelet interactions in normal and HIT systems
i.v. and s.c. half-life about 14 hr	i.v. half-life about 90 min
100% bioavailable s.c.	Orally bioavailable
Limited bleeding side effect	Limited bleeding side effect
Predictable dose response	Predictable dose response

HIT, heparin-induced thrombocytopenia.

Antithrombotic actions of DX-9065a were also demonstrated after oral administration of this agent (45,50-52). After an intravenous dose of 1 mg/kg, significant anticoagulant and anti-factor Xa activities could be measured in plasma up to 120 minutes in both rabbits and primates (Figs. 10-6 and 10-7).

DX-9065a in global clotting assays caused a significant dose-dependent prolongation of the APTT, the prothrombin time test (PT), and the ACT (Fig. 10-2) both in *in vitro* and *ex vivo* plasma (43,45,48,50-52). The PT assay was more sensitive to the anticoagulant effect of DX-9065a (Fig. 10-6). DX-9065a does not

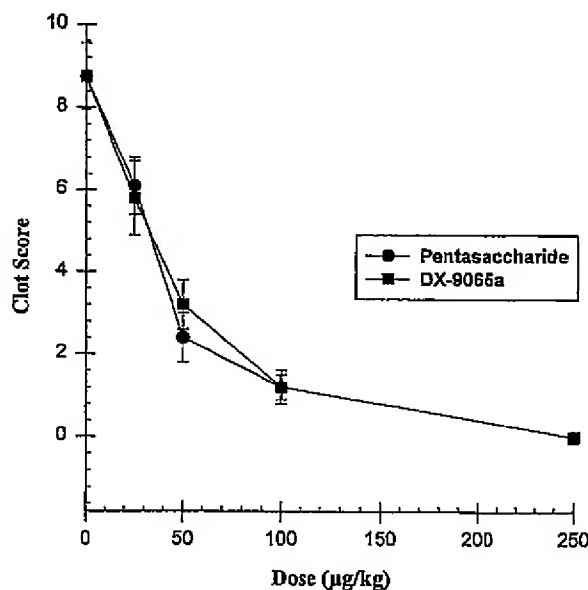


FIG. 10-5. Comparative study of the antithrombotic effect of DX-9065a and pentasaccharide in a modified Wessler rabbit stasis thrombosis model using FEIBA as a thrombogenic stimulus. At equigravimetric dosages, comparable antithrombotic activity was achieved with both agents. Thus direct and indirect acting factor Xa inhibitors are capable of producing inhibition of induced venous thrombosis.

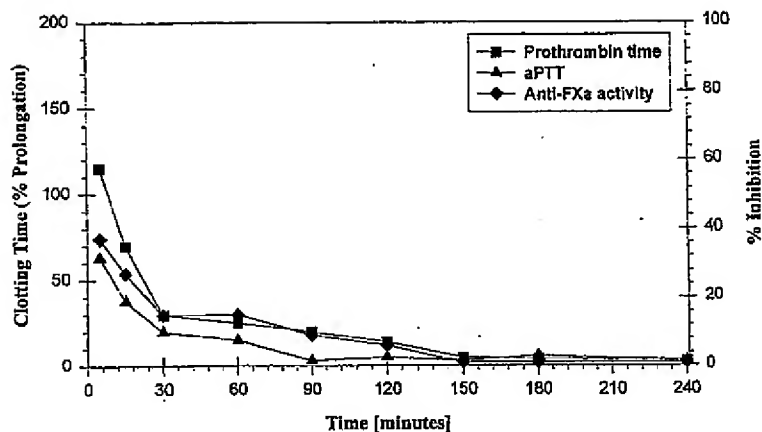


FIG. 10-6. Anticoagulant and anti-factor Xa activities of DX-9065a after administration of a 1 mg/ intravenous dosage to rabbits (n = 5).

have any effect on preformed thrombin; however, the inactivation of factor Xa by DX-9065a can prevent the further generation of thrombin with resulting inhibition of thrombin-mediated feedback reactions. The formation of thrombin via the intrinsic pathway was more effected than the extrinsic thrombin

generation (43). The inhibition of thrombin generation was found in ex vivo samples after intravenous injection of DX-9065a into rabbits (43).

For unknown reasons the anticoagulant action of DX-9065a is species dependent, showing much less activity in rat, dog, and monkey plasma than in human and common squirrel monkey plasma (48,55).

Pharmacokinetic studies on DX-9065a showed a biologic half-life of about 6 minutes for the α phase and 99 minutes for the β phase when given intravenously (50). Oral administration produced peak plasma concentration at 30 minutes, and plasma levels as well as anticoagulant effects gradually declined over about 6 to 8 hours (45,50). The bioavailability after oral administration was estimated to approximately 5% to 12% (50).

DX-9065a does not compromise the hemostatic reaction of platelets. It does not affect platelet aggregation (45) and does not produce a positive platelet aggregation effect in heparin-induced thrombocytopenia positive system in vitro (J. Fareed, unpublished data). As a competitive inhibitor of factor Xa, it does not completely suppress the production of thrombin. Small amounts of thrombin generated despite factor Xa inhibition can initiate primary hemostasis by forming platelet

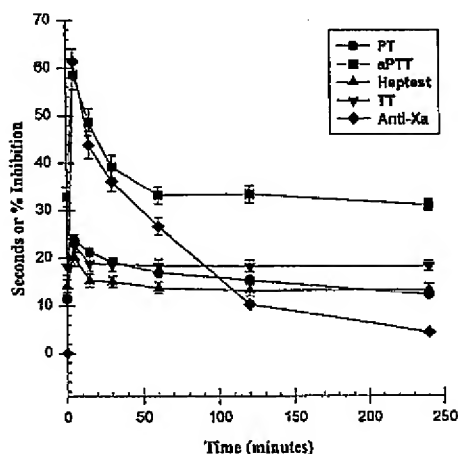


FIG. 10-7. Anticoagulant and anti-factor Xa activities of DX-9065a after administration of a 1-mg/kg intravenous dose to nonhuman primates (*Macaca mulatta*) (n = 4).

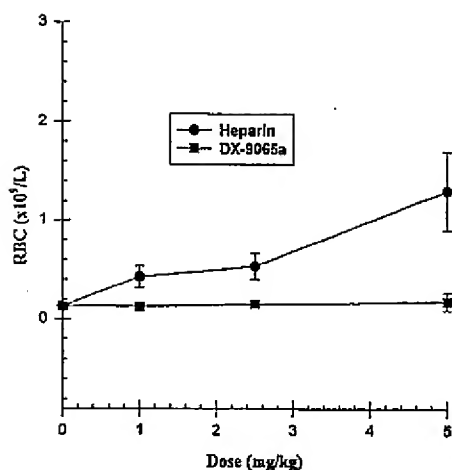


FIG. 10-8. Comparative studies on the bleeding effect of heparin and DX-9065a. The relative hemorrhagic effects of DX-9065a and heparin in a rabbit ear bleeding model show a markedly weaker effect of the factor Xa inhibitor in comparison with heparin. Even at a supratherapeutic dose of 5 mg/kg, the bleeding effect of DX-9065a was insignificant. Heparin, on the other hand, produced a dose-dependent effect. Thus, DX-9065a and most likely other factor Xa inhibitors have a wider therapeutic index than heparin, rendering them safer antithrombotic agents.

mostatic plugs. However, this small amount of thrombin would be insufficient to catalyze the conversion of fibrinogen to fibrin.

DX-9065a has nearly no effect in a rabbit ear bleeding model at doses higher than those shown to be effective for antithrombotic protection (Fig. 10-8). DX-9065a has been shown to have a favorable safety index regarding bleeding complications in animals (43,45,51).

Selectide Series

The Selectide (SEL) (Selectide Corp., Tucson, AZ) series of agents are pentapeptides produced by combinatorial chemistry (56). They are in the preclinical phase of development.

YM-60828

YM-60828 is an orally active agent in preclinical development. In animal models it has

been shown to be effective against carotid arterial thrombosis and as an adjunct to thrombolysis after oral administration (57).

Summary

1. Numerous direct factor Xa inhibitors are under development as antithrombotic agents. These agents bind factor Xa and do not require any plasma cofactors. They can also inhibit clot-bound factor Xa.
2. TAP, a recombinant derived agent, is a selective and potent factor Xa inhibitor that has shown promise in experimental models of thrombosis. It is also being studied as an inhibitor of restenosis after angioplasty.
3. TFPI, a recombinant derived agent, is a natural human plasma-based anticoagulant. It is in human clinical trials.
4. DX-9065a is a synthetic factor Xa inhibitor with potent activity. It does not show bleeding effects in experimental models at antithrombotic dosages. It is in human clinical trials.

INDIRECT INHIBITORS

Heparin Pentasaccharide

The only available indirect factor Xa inhibitor is the heparin pentasaccharide. This agent produces its antithrombotic effect via high-affinity binding to AT III. It is the smallest heparin-based molecule (molecular weight 1,728 daltons) that retains antithrombotic activity, being composed of five saccharide units, two of the regular region of heparin and three of the irregular region (Fig. 10-9) (58). Pentasaccharide was developed in 1983 as proof of the hypotheses that a five-member heparin chain was the most minimum saccharide sequence for antithrombotic activity and that sole factor Xa inhibition was indeed antithrombotic (59-61). It was shown in experimental models that inhibition of factor Xa controls excessive thrombin generation, produces antithrombotic effect and has less bleeding risk than heparin (62-64).

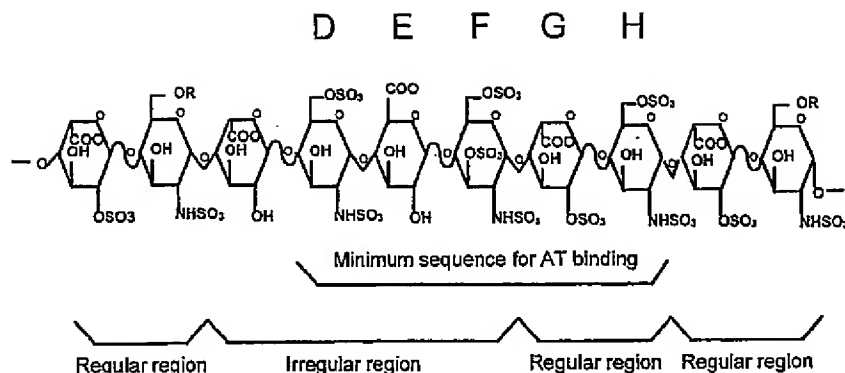


FIG. 10-9. Structure of the synthetic heparin pentasaccharide. (From Walenga et al., ref. 58, with permission.)

The heparin pentasaccharide is produced synthetically in a cooperative effort by Sanofi and Organon. Once the chemical synthesis process was established (60,61), other related agents that were structural modifications of the first pentasaccharide were synthetically produced. Of all materials synthesized, it was determined that the binding to AT III was most critical for expression of antithrombotic activity (65-68). These agents have stronger anti-factor Xa potencies and longer half-lives than the first synthetic pentasaccharide (65,68-70). Pentasaccharide is in phase II clinical trials for prophylaxis against venous thrombosis after hip and knee replacement; none of the modified agents are in clinical trial yet.

Because of its small size, it possesses anti-factor Xa activity with no inhibitory actions against thrombin or other serine proteases. The pentasaccharide-AT III complex has a potency of about 700 anti-factor Xa IU/mg in human plasma and 360 anti-factor Xa IU/mg in rabbit plasma (67). An equivalent amount of AT III is required for complete expression of the anti-factor Xa activity of pentasaccharide (71). Recently Lormeau et al. showed that pentasaccharide inhibits the coagulant activity of the factor VIIa-tissue factor complex (72). The antithrombotic activity was found to be related to the inhibition of

thrombin generation shown in *in vitro* and *in vivo* settings, but the maximal inhibition was less than that for heparin (63,73-75). The same result was obtained after administration of pentasaccharide to humans (76). Thrombin generation inhibition after extrinsic pathway activation was stronger than the inhibition after intrinsic pathway activation.

The APTT was minimally affected (about 100 seconds at a very high dose) by pentasaccharide, and the PT and ACT (Fig. 10-2) were not affected at all (48,58). No platelet interactions have been observed for pentasaccharide in agonist-induced systems for aggregation or in heparin-induced thrombocytopenia test systems (77).

Pentasaccharide has shown dose-dependent antithrombotic activity in several animal models of thrombosis (Fig. 10-5), the degree of antithrombotic activity being dependent on the thrombogenic stimulus for venous thrombosis (40,62,64,78). Pentasaccharide was also effective against arterial thrombosis in a rat arteriovenous shunt model (64,79,80), in a modified arteriovenous shunt model in baboons (81), and in a laser-induced rat model (58,80). This agent also has been shown to facilitate fibrinolysis induced by tissue plasminogen activator in rabbits (82).

Pharmacokinetic studies show a prolonged half-life in both intravenous (approximately

hours) and subcutaneous (approximately 18 hours) dosing regimens (64,70,83). Subcutaneous bioavailability was near 100% (84). Human pharmacokinetics showed a similar half-life at about 13.5 hours and a linear correlation with dose (85). The majority of pentasaccharide was eliminated through the kidneys. In elderly subjects the half-life was prolonged to 14.5 hours, and plasma clearance was decreased (85). Bleeding studies in rats suggested very minor bleeding at dosages 100-fold higher than required for complete protection against induced thrombosis (58,64).

Clinical trials are in progress for the prophylaxis of venous thrombosis in orthopedic surgical patients. A brief study on the successful use of pentasaccharide with percutaneous transluminal coronary angioplasty has recently been reported (86). Pentasaccharide is more attractive than LMW heparins because it is a well-defined synthetic drug with a long half-life and 100% subcutaneous bioavailability. It does not appear to be related to the heparin-induced thrombocytopenic response and therefore may be used as a substitute antithrombotic agent in patients with heparin-induced thrombocytopenia (87). It appears to have minimal bleeding risks.

Summary

1. The heparin pentasaccharide is the only well-developed indirect factor Xa inhibitor. Its structure is based on heparin and it requires AT III to express activity. It is a well-defined synthetic agent.
2. Pentasaccharide has a long half-life (intravenous or subcutaneous). It has been shown to be antithrombotic in experimental models with no bleeding side effect.
3. Clinical trials of pentasaccharide as prophylaxis against thrombosis after hip and knee replacement surgery are nearing completion. If successful, the hypothesis that factor Xa inhibition is an effective means to produce antithrombotic activity will be validated.

CONCLUSION

Factor Xa plays a pivotal role in the coagulation process, being the common point between the extrinsic and intrinsic pathways. Although thrombin is an important enzyme, its generation is dependent on factor Xa. Thus, to control the activity of activated factor X would be to control excessive thrombin generation. With less thrombin, the rate of fibrin formation is slowed. A regulated control of the activation of the coagulation cascade would be achieved. With factor Xa inhibitors, bleeding risk would be minimal because some thrombin generation/clot formation is still possible under treatment because thrombin generation is not completely blocked.

The factor Xa inhibitors presently under clinical development are a diverse class of new antithrombotic agents with direct and indirect mechanisms of inhibition. The pentasaccharide represents a synthetic oligosaccharide that requires the endogenous cofactor AT III for its activity. TAP is a LMW peptide with direct inhibitory activity, whereas DX-9065a is a synthetic organic compound of lower molecular weight and direct inhibitory actions. DX-9065a is less specific than TAP. TFPI is also a promising agent.

Despite differences in their mechanisms of action and in vitro activities, pentasaccharide, DX-9065a, and TAP have been shown to be effective antithrombotic agents in experimental models of venous thrombosis, coronary artery occlusion, arterial thrombolysis and acute re-occlusion, restenosis after angioplasty, dialysis, and disseminated intravascular coagulation (DIC). Preliminary results from human trials in orthopedic surgical patients are encouraging. Both TAP and DX-9065a produce measurable in vitro anticoagulant effects. In contrast, pentasaccharide does not produce an anticoagulant effect by the typical clot-based assays. Thus, with factor Xa inhibitors there is not necessarily a correlation between current laboratory assays and antithrombotic efficacy as there is with heparin.

Factor Xa inhibitors vary in their efficacy to inhibit factor Xa depending on molecular

11

Unfractionated and Low Molecular Weight Heparin

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UNFRACTIONATED HEPARIN

Mechanism of Action

Unfractionated heparin is a glycosaminoglycan (GAG) composed of chains of alternating residues of D-glucosamine and a uronic acid (1). Its major anticoagulant effect is accounted for by a unique pentasaccharide with a high-affinity binding sequence to antithrombin (AT), which is present in only one third of unfractionated heparin molecules

(8-11). Heparin binds to AT (2-11) and produces a conformational change in AT (12-14) that markedly accelerates its ability to inactivate the coagulation enzymes thrombin (factor IIa), factor Xa, and factor IXa (3). After AT binds to form an irreversible complex with these coagulation enzymes, heparin dissociates from the complex and can be reutilized. Of the coagulation enzymes inactivated by the heparin-AT complex, thrombin is the most sensitive to inhibition (3,15-19).

Heparin catalyzes the inactivation of thrombin by AT by acting as a template to which both the enzyme and inhibitor bind to form a ternary complex (3,11,20,21). In contrast, the inactivation of factor Xa by AT-heparin complex does not require ternary complex formation and is achieved by binding of the enzyme to AT (1,3,6,7). Unfractionated heparin molecules that contain fewer than 18 saccharides are unable to bind thrombin and AT simultaneously and, therefore, are unable to accelerate the inactivation of thrombin by AT, but retain their ability to catalyze the inhibition of factor Xa by AT (21-23). Heparin also catalyzes the inactivation of thrombin by a second plasma cofactor, heparin cofactor II (HCII) (24). This second anticoagulant effect of unfractionated heparin is specific for thrombin, does not require the unique AT-binding pentasaccharide, and requires much higher doses of heparin (25-28) than those required to catalyze the activity of AT.

Unfractionated heparin is heterogeneous with respect to molecular size, anticoagulant activity, and pharmacokinetic properties. The molecular weight of unfractionated heparin ranges from 5,000 to 30,000 daltons with a mean molecular weight of 15,000 daltons (approximately 50 monosaccharide chains) (29-31). The anticoagulant activity of unfractionated heparin is heterogeneous because (a) only one third of the unfractionated heparin molecules administered to patients have anticoagulant activity; (b) the anticoagulant profile of unfractionated heparin is influenced by the chain length of the molecules; and (c) the clearance of unfractionated heparin is influenced by its molecular size, with the higher molecular weight species being cleared from the circulation more rapidly than the lower molecular weight species.

Administration, Pharmacokinetics, and Pharmacodynamics

Heparin must be given by injection; the two preferred routes are intravenous and subcutaneous. The efficacy and safety of unfractionated heparin administered by either the con-

tinuous intravenous method or by the subcutaneous route are comparable provided the dosages used are adequate (32-34). When administered subcutaneously in high dose the bioavailability of unfractionated heparin is reduced by about 10% (compared with heparin administered by continuous intravenous infusion), and its anticoagulant effect is delayed for 1 to 2 hours.

Unfractionated heparin binds to a number of plasma proteins, which contributes to reduced plasma recovery (bioavailability) at low concentrations, to the variability of the anticoagulant response to fixed doses of heparin in patients with thromboembolic disorders (35), and to the laboratory phenomenon of heparin resistance (36). Binding of heparin to von Willebrand factor (VWF) results in inhibition of VWF-dependent platelet function (37).

Heparin also binds to macrophages and endothelial cells (38). Unfractionated heparin is cleared through a combination of a rapid saturable and a much slower first-order mechanism of clearance (39-41). The saturable phase of heparin clearance is thought to be due to heparin binding to receptors on endothelial cells (42,43) and macrophages (44) where it is internalized, depolymerized, and metabolized into smaller and less sulfated forms (45,46). Clearance through the slow nonsaturable mechanism is partly renal. At therapeutic doses, a considerable proportion of the administered unfractionated heparin is cleared through the rapid saturable, dose-dependent mechanism of clearance. The apparent biologic half-life of unfractionated heparin increases from approximately 15 minutes with an intravenous bolus of 25 U/kg to 60 minutes with an intravenous bolus of 100 IU/kg, to 150 minutes with an intravenous bolus of 400 U/kg (39-41).

LABORATORY MONITORING AND DOSE-RESPONSE RELATIONSHIPS OF HEPARIN

The anticoagulant effect of unfractionated heparin is usually monitored by the activa-

bound thrombin may result from conformational changes in the active site of thrombin that occur when the enzyme binds to fibrin. The marked resistance of fibrin-bound thrombin to the heparin-AT complex occurs because the heparin binding site on thrombin (so-called exosite 2) is masked when thrombin binds to fibrin. The explanation for the protection of factor Xa from inactivation by heparin-AT is less well understood, but may reflect similar mechanisms. The inability of heparin to inactivate surface-bound thrombin and factor Xa may explain why heparin is of only limited efficacy in cardiopulmonary bypass surgery, unstable angina, high-risk coronary angioplasty, and coronary thrombolysis.

Biologic Limitations

The biologic limitations of heparin reflect its propensity to bind to platelets and to activate them (146). Platelet activation can contribute to bleeding because degranulated platelets have impaired hemostatic function. In addition, platelet factor 4 released from activated platelets can complex with heparin, thereby triggering the formation of the antibodies that cause heparin-induced thrombocytopenia (HIT) (147).

Low molecular weight heparins overcome the pharmacokinetic and biologic limitations of unfractionated heparin and share the same biophysical limitations. The biophysical limitations of both low molecular weight heparins and unfractionated heparins have provided an important rationale for the development of new antithrombotics.

LOW MOLECULAR WEIGHT HEPARINS

Low molecular weight heparins are a new class of anticoagulants which are replacing unfractionated heparin for many indications in Europe and are being used for more limited indications in North America.

Low molecular weight heparins are derived from unfractionated heparin by either chemical or enzymatic depolymerization to yield fragments that are approximately one third the

size of heparin. Like heparin, they are heterogeneous with respect to molecular size : anticoagulant activity. Low molecular weight heparins have a mean molecular weight 4,000 to 5,000 daltons with a molecular weight distribution of 1,000 to 10,000 daltons. Depolymerization of heparin into low molecular weight fragments results in the main changes in the properties of heparin. Thus, the resultant low molecular weight heparins have (a) a change in their anticoagulant profile with a progressive loss of their ability to catalyze thrombin inhibition (11,21,148); (b) reduced protein binding with an improvement in their pharmacokinetic properties (29,38,149-153); and (c) reduced interaction with platelets (146), which could be responsible for the reduced microvascular bleeding in experimental animal models and the lower incidence of HIT (154). Two other cosaminoglycans also have been developed for clinical use. These are dermatan sulfate and the heparinoid danaparoid sodium, which is a mixture of heparin sulfate (the major component making up 80% of the mixture) and smaller amounts of dermatan sulfate and chondroitin sulfates.

It should be noted that commercially available low molecular weight heparins are prepared using different methods of depolymerization; therefore, they are not necessarily clinically interchangeable.

Anticoagulant Effects of Low Molecular Weight Heparins

Like unfractionated heparin, low molecular weight heparins produce their major anticoagulant effect by binding to AT via a unique pentasaccharide sequence (6,7), which is present on less than one third of low molecular weight heparin molecules. Because a minimum chain length of 18 saccharides (including the pentasaccharide sequence) is required for ternary complex formation, only the top 50% of low molecular weight heparin species that are above this critical length in the different commercial low molecular weight heparin preparations are able to inactivate thrombin. In contrast, all of the

molecular weight heparin fragments that contain the high-affinity pentasaccharide catalyzes the inactivation of factor Xa. Virtually all unfractionated heparin molecules contain at least 18 saccharide units (155,156). Therefore, in contrast to unfractionated heparin, which has a ratio of anti-factor Xa to anti-factor IIa activity of approximately 1:1, the various commercial low molecular weight heparins have anti-factor Xa to anti-factor IIa ratios that vary between 4:1 and 2:1, depending on their molecular size distribution.

Pharmacokinetics of Low Molecular Weight Heparins

The plasma recoveries and pharmacokinetics of heparin and low molecular weight heparins differ because of differences in their relative-binding properties to plasma proteins and cells. Most heparin-binding proteins do not bind to or neutralize low molecular weight heparins (29,38,149-151,153). The absence of protein binding of low molecular weight heparins contributes to their excellent bioavailability at low doses (157) and to their more predictable anticoagulant response when administered in fixed doses (158). Low molecular weight heparin preparations also have a lower affinity than heparin for VWF (38), a property that could contribute to the observation that low molecular weight heparins produce less experimental bleeding than heparin for equivalent anticoagulant effects (159-165). Unlike heparin, low molecular weight heparins do not bind to endothelial cells in culture (39,166,167), a property that could be responsible for their longer plasma half-life (which is approximately two- to fourfold longer than that of heparin) (168-174). Low molecular weight heparins are cleared principally by the renal route, and their biologic half-life is increased in patients with renal failure (168,175,176).

Antithrombotic and Hemorrhagic Effects of Low Molecular Weight Heparins, Heparinoids, and Unfractionated Heparin in Experimental Models in Animals

The antithrombotic effects and hemorrhagic effects of heparin have been compared

with low molecular weight heparins with the heparinoid danaparoid sodium and with dermatan sulfate in a variety of experimental animal models (159-165,177-181). In these models of thrombosis, temporary venous stasis is produced by ligating an appropriate vein, and blood coagulation is stimulated by injecting either serum, factor Xa, thrombin, or tissue factor (165,180,181). When compared on a gravimetric basis, low molecular weight heparins are slightly less effective than heparin as antithrombotic agents but produce much less bleeding than heparin in models measuring blood loss from a standardized injury (160-163,165,178,179).

These differences in the relative antithrombotic to hemorrhagic effects of these polysaccharides could be due in part to their different effects on platelet function (38,182,183) and vascular permeability (184).

Arterial Thrombosis

Low molecular weight heparins have been evaluated in a canine model of coronary artery thrombosis (185). Low molecular weight heparin in a dose of 2.5 mg/kg subcutaneously was as effective as unfractionated heparin in a dose of 10 mg/kg subcutaneously (185). A second study compared the relative efficacy and safety of a very low molecular weight heparin (CY222) with unfractionated heparin in an exteriorized femoral arteriovenous shunt in baboons (186). Skin bleeding times also were measured. In this platelet-dependent model, low molecular weight heparin demonstrated a more favorable antithrombotic to bleeding ratio than did unfractionated heparin.

Clinical Experience with Low Molecular Weight Heparin Preparations

Low molecular weight heparins have been evaluated in a large number of randomized clinical trials and have been shown to be safe and effective anticoagulants for the prevention and treatment of venous thrombosis. More recently, low molecular weight heparin preparations have been evaluated in patients

with unstable angina (187,188) and with stroke (189), postfemoropopliteal arterial surgery (190), and for the prevention of restenosis after angioplasty (191).

The largest experience with low molecular weight heparin has been obtained in the prevention of venous thrombosis in high-risk patients. Experience with low molecular weight heparins for the treatment of venous thrombosis is growing, whereas studies evaluating low molecular weight heparins in arterial thrombosis are in their early stages.

Although the low molecular weight heparins in clinical use have many similarities, they also differ from one another in molecular weight distribution profiles, in their relative specific activities (anti-Xa to anti-IIa activities), in their rates of plasma clearance, and in their recommended dosage regimens and in experimental microvascular bleeding (Table 11-1). Therefore, it cannot be assumed that results obtained for a specific indication using one low molecular weight heparin would also be obtained with another low molecular weight heparin.

Prevention of Venous Thrombosis

The results of studies evaluating low molecular weight heparins for the prevention of venous thrombosis have been reviewed elsewhere (192).

For general surgical patients, low molecular weight heparins administered once daily by subcutaneous injection have been shown to reduce cardiovascular mortality when compared with placebo and to be approximately 30% more effective than unfractionated heparin 5,000 IU (administered by subcutaneous injection twice or three times daily) in preventing venous thrombosis without any difference in bleeding (192).

When compared with a control group, low molecular weight heparins have been shown to reduce the incidence of thrombosis in patients having major knee or hip surgery by about 70% without increasing the risk of bleeding (193-195). When compared directly with other forms of prophylaxis in orthopedic

patients, low molecular weight heparins are significantly more effective than heparin 5,000 IU subcutaneously administered twice or three times daily (196-198), significantly more effective than oral anticoagulants (199-201), significantly more effective than dextran (202,203), significantly more effective than aspirin (204), and significantly more effective than adjusted-dose heparin in preventing proximal vein thrombosis (205).

Low molecular weight heparins are very effective in preventing venous thrombosis in patients with thrombotic stroke (206,207) and other high-risk medical patients (208), producing a relative risk reduction in venous thrombosis of between 60% and 90%. This beneficial effect occurred without an increase in clinically important bleeding. Low molecular weight heparins also have been shown to be significantly more effective than unfractionated heparin in preventing venous thrombosis in patients with paralytic stroke and in patients with spinal cord injury. Thus, in both studies comparing low molecular weight heparins with heparin, patients randomized to receive low molecular weight heparin showed a greater than 70% risk reduction in thrombosis, a statistically significant difference (209,210).

TREATMENT OF VENOUS THROMBOSIS

Treatment of Established Thrombosis

There is growing experience with the use of low molecular weight heparins for the treatment of venous thrombosis. Low molecular weight heparin has been compared with unfractionated heparin in the treatment of venous thrombosis (Table 11-2). In the major studies, low molecular weight heparin was administered by subcutaneous injection (usually twice daily) and unfractionated heparin was administered by continuous intravenous infusion with APTT monitoring.

Eight trials (211-218) compared the effect of heparin and low molecular weight heparin by measuring the change in thrombus size after 5 to 10 days of treatment observed in pretreatment and posttreatment venogra-

TABLE 11-1. The anticoagulant profiles, molecular weights, plasma half-lives, and recommended doses of commercial low molecular weight heparins

Agent	Anti-Xa to anti-IIa ratio	Molecular weight (range) [saccharide units]	Plasma half-life (min)	Recommended dose (converted into International anti-Xa units)		Treatment
				General surgery	Orthopedic surgery	
Enoxaparin sodium (Lovenox/Clexane) (Rhône-Poulenc Rorer)	2.7:1	4,500 (3,000–8,000) [10–27]	129–180	2,000 U s.c.	4,000 U s.c. daily or 3,000 U s.c. BID	7,000 U s.c. BID ^a
Dalteparin (Fragmin) (Kabli)	2.0:1	5,000 (2,000–9,000) [7–30]	119–139	2,500 U s.c.	2,500 U s.c. BID or 5,000 U s.c. daily	8,400 U s.c. BID ^a
Nadroparin calcium (Fraxiparin) (Sanofi)	3.2:1	4,500 (2,000–8,000) [7–27]	132–162	7,500 U/I.C. s.c. daily		31,500 U/I.C. daily ^a
Innohep (Tinzaparin) (Leo Laboratories)	3.2:1	4,500 (2,000–8,000) [7–27]	132–162	7,500 U/I.C. s.c. daily		31,500 U/I.C. daily ^a
Ardeparin (Normflo) (Wyeth-Ayerst)	1.9:1	4,500 (3,000–6,000) [10–20]	111	3,500 U s.c. daily	50 U/kg s.c. daily	12,250 daily ^a
Danaparoid sodium ^b (Orgaran) (NV Organon)	2.0:1	6,000 (2,000–15,000) [7–50]	200		50 U/kg s.c. BID	
	20:1	6,500	1,100		750 U s.c. BID	1,250 U s.c. BID

^aWeight-adjusted dose; stated dose for 70 kg patient.

^bDanaparoid sodium (Orgaran) is a heparinoid.

U/I.C., Institute Choay Units; 3 ICU, 1 International Unit.

Other Antithrombotics

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Thrombomodulin	251	Inhibitors of Factor VIII	259
Protein C	252	Naroparcil	259
Defibrinolytic	254	Coagulation Inhibitors Derived from	
Tissue Factor Pathway Inhibitor	255	Aprotinin	259
Inactivated Tissue Factor	257	Inhibitors of Factor XIII	260
Blockers of the Thrombin Receptor Function	257	Conclusion: The Ideal Antithrombotic	
Human Plasma-Derived and Recombinant		Drug	260
Antithrombin III	258		

The previous six chapters describe the advantages but also the limitations of different classes of antithrombotic drugs. Substantial research is focusing on other compounds with antithrombotic properties, which are briefly discussed here.

THROMBOMODULIN

Thrombomodulin is an endothelial cell surface protein that forms a reversible 1:1 stoichiometric complex with thrombin. After formation of this complex, thrombin no longer has procoagulant activity but does acquire the potential to activate protein C 1000-fold compared to free thrombin (1-3). The term *thrombomodulin* is appropriate because this protein changes the substrate specificity of thrombin, apparently by an allosteric mechanism. Activated protein C, in the presence of protein S, inactivates blood coagulation factors Va and VIIIa (4,5). Thus, by accelerating the activation of protein C, thrombomodulin plays an important role as endogenous regulator of coagulation at the surface of the vascular wall.

Furthermore, thrombomodulin inhibits the proteolytic action of thrombin on macromolecular substrates, and because thrombomodulin contains a galactosaminoglycan, it accelerates the inactivation of thrombin by antithrombin III.

The thrombin-thrombomodulin complex is also involved in the regulation of fibrinolysis as it activates another macromolecular substrate, termed Thrombin Activatable Fibrinolysis Inhibitor (TAFI), by cleavage of a single proteolytic cleavage (6,7). The enzyme, TAFIa, is a carboxypeptidase with specificity for carboxy terminal arginine and lysine residues (8) and is a potent inhibitor of fibrinolysis (6,9,10). This suppression of fibrinolysis is most likely obtained by downregulation of the cofactor function of partially degraded fibrin (removing plasminogen binding sites). The existence of TAFI explains the apparent profibrinolytic effect of activated protein C.

The lethal effect of deletion of thrombomodulin gene in the mouse was demonstrated (11). Thrombomodulin mutation cosegregates with thromboembolic disease (12).

Thrombomodulin is an integral membrane glycoprotein containing 575 amino acids and 5 domains. It is present on the vascular surface of endothelial cells of arteries, veins, capillaries, and lymphatic vessels (Fig. 14-1). Thrombomodulin purified from human urine has a potent antithrombotic effect in the rat disseminated intravascular coagulation model (13). The human thrombomodulin cDNA has been isolated (4) and expressed in Chinese hamster ovary (CHO) cells (14). This soluble human thrombomodulin contains only the domains 1, 2, and 3 (amino acids 1-491) but not the transmembrane module and cytoplasmic tail of native single-chain thrombomodulin.

Recombinant human soluble thrombomodulin is effective in the rat model of arteriovenous shunt thrombosis (15) and in disseminated intravascular coagulation models in mice and rats, also when the levels of antithrombin III are reduced. The presence of chondroitin sulfate on recombinant human soluble thrombomodulin results in a higher affinity for thrombin, a greater ability to inhibit thrombin-induced fibrin formation and

platelet activation, and is associated with antithrombin III-dependent inactivation of thrombin (16,17). Administration of thrombomodulin prolongs the thrombin time (thrombin time (TT)), prothrombin time (PT), and activated partial thromboplastin time (APTT). Recombinant human thrombomodulin may be a means to generate endogenous activated protein C.

The parenteral administration of soluble recombinant thrombomodulin has been associated with antithrombotic effects without bleeding in cancer patients (18).

PROTEIN C

Activated protein C is a natural coagulation inhibitor that plays a key role in the regulation of blood coagulation by selectively degrading coagulation cofactors Va and VIIIa, thereby inhibiting thrombus generation. Thus, activated protein C stops the positive feedback actions of thrombin on the coagulation cascade (thrombin activates factors II and V), thereby limiting the coagulation process and thrombus propagation (Fig. 1

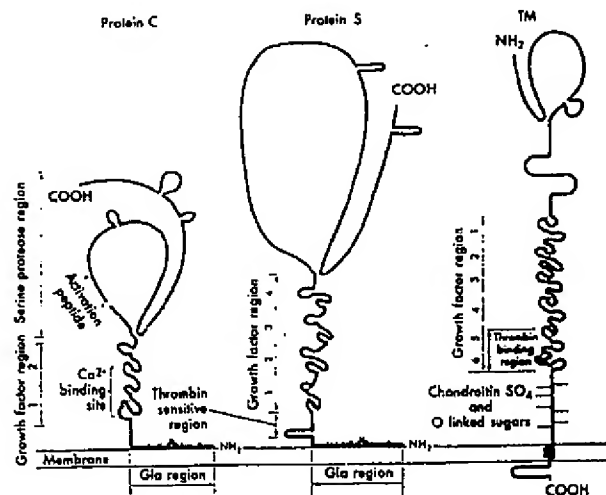


FIG. 14-1. Schematic representation of protein C, protein S, and thrombomodulin (TM). The residues (γ-carboxyglutamic acid) are indicated as small Y-shaped symbols on protein C and protein S. These residues are required for biological activity and depend on vitamin K for their biosynthesis. (From Esmon CT, ref. 3, and Esmon ML, ref. 107, with permission.)

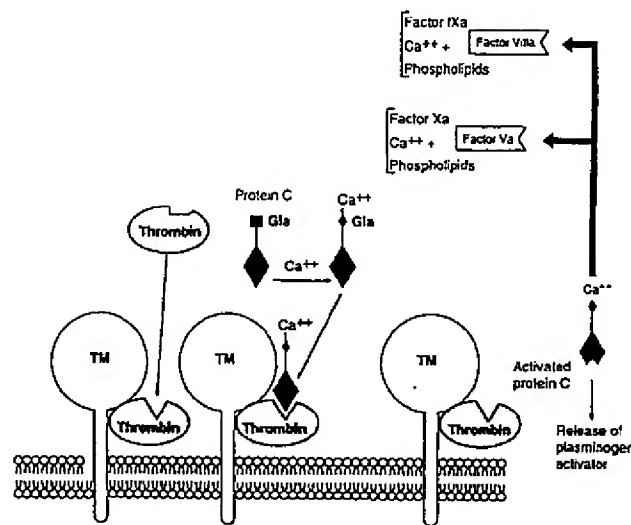


FIG. 14-2. Thrombin forms a complex with the endothelium-bound protein thrombomodulin (TM). This complex activates circulating protein C, which inhibits factor Va and VIIIa and releases tissue plasminogen activator from the endothelial cells. Binding of activated protein C to phospholipids is facilitated by protein S. Gla, γ -carboxyglutamic acid.

Protein C is one of the vitamin K-dependent plasma proteins; it is activated on the surface of intact endothelial cells by thrombin bound to thrombomodulin. The anticoagulant effect of activated protein C is enhanced by protein S, which is another vitamin K-dependent plasma protein (20). It has been reported that protein S increases the affinity of activated protein C for thrombogenic phospholipids approximately 10-fold and that protein S abrogates the protective effect of factor Xa against activated protein C-mediated degradation of factor Va in the prothrombinase complex.

An endothelial cell protein C receptor has been identified (21). It is a transmembrane protein that is expressed at high levels only on a subset of endothelial cells. These receptors augment protein C activation. Binding to the receptor is Ca^{2+} -dependent and is mediated in part by the Gla domain of the protein.

Human plasma contains 4 mg/L of protein C. The protein can be purified from plasma or obtained by recombinant technology (22). Because of its endogenous origin and specificity

of action, activated protein C is a potentially attractive antithrombotic agent. Earlier studies have shown that activated protein C inhibits disseminated intravascular coagulation in rabbits (23) and blocks the lethal effects of *Escherichia coli* infusion in baboons (24). Moreover, human activated protein C has been shown to reduce jugular vein thrombus formation in dogs, to delay the time to occlusion in anodal current-induced rat aorta thrombosis (25), and to reduce intermittent platelet thrombus formation in rat microvessels (26). Human activated protein C inhibits thrombus formation on thrombogenic grafts interposed in arteriovenous shunts in baboons, when infused into the shunt just proximal to the thrombogenic site (27).

Using a bovine protein C preparation in a microarterial thrombosis model in the rabbit, antithrombotic efficacy was demonstrated but was associated with significant bleeding (28).

Thrombin mutants with selectivity for endogenous protein C have been developed (29, 30). Activated protein C administration does

not impair hemostasis (31), which supports the proposal to either use protein C or increase its plasmatic level by administering thrombin mutants without affecting the fibrinogen conversion.

DEFIBROTIDE

Defibrotide is the sodium salt of a single-strand polydeoxyribonucleotide (aptamers) of a well-defined base sequence and composition that binds to thrombin. The compound has a mean molecular weight of 15 to 30 kDa with a defined ratio of purine to pyrimidine bases of >0.85 . It is prepared by controlled depolymerization of deoxyribonucleic acid (DNA) obtained from porcine organs.

In most studies, particularly those using a three- to fourfold higher dose than that recommended for patients, defibrotide stimulates PGI_2 and PGE_2 production without change in thromboxane A_2 levels and is associated with reduced leukotriene B_4 levels (32). The drug also stimulates the fibrinolytic system, as shown by a decrease of the euglobulin lysis time and dilute clot lysis time. Furthermore, the lysis area of euglobulin on standard fibrin plates increases (33,34). There are conflicting observations regarding the effect of defibrotide on activity in blood of tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor type 1 (PAI-1), and α_2 -antiplasmin (35).

Defibrotide appears to be largely devoid of anticoagulant properties as determined by a lack of clinically significant effects on coagulation parameters, including APTT and the prothrombin time. The drug appears to have no effect on von Willebrand factor, factor VIII, factor Xa, and prekallikrein, whereas its effect on antithrombin III, fibrinogen, and protein C requires further confirmation.

Defibrotide has been reported to have little effect on platelet numbers, but may inhibit platelet function, possibly by stimulating the formation of PGI_2 and blocking calcium ions' entry into cells by interfering with the adenosine receptor (36). It has recently been shown that defibrotide stimulates expression of

thrombomodulin in cultured human umbilical vein endothelial cells (37).

Investigation of the pharmacokinetic behavior of defibrotide is difficult because drug is degraded in the body to a number of products and the identity of the active derivative(s) in humans is unclear. To date, the majority of pharmacokinetic data have been determined by following the fate of carbohydrate moiety of the drug 6-deoxybose. Elimination of defibrotide in humans follows different kinetic models depending on the dose, with a one-compartment model being the most appropriate following administration of low doses, and a two-compartment model better suited following high doses. Defibrotide elimination half-life is short and increases with dose, with values of between 9.8 and 27.1 minutes after intravenous doses of 0.16 mg/kg or a single intravenous injection of 200 mg. The elimination half-life appears to be independent of the route of administration with similar values being obtained after oral and intravenous administration.

An antithrombotic action of defibrotide has been demonstrated in a number of animal models of venous thrombosis, in which the

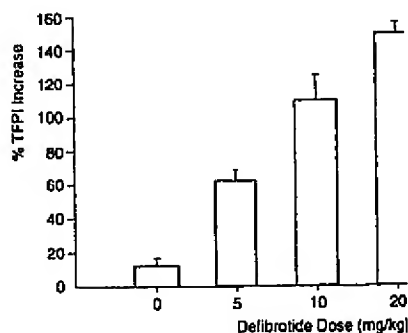


FIG. 14-3. Effect of oral administration of defibrotide on plasma tissue factor pathway inhibitor (TFPI) levels in normal healthy volunteers. Defibrotide was administered at 5, 10, and 20 mg/kg dosages to groups of healthy individuals ($n=10$). Blood samples were drawn 4 hours after administration of defibrotide. (From Fareed et al., ref. 108, with permission.)

was able to attenuate the formation of collagen-, activated prothrombin complex-, thrombin-, and mechanically induced venous thrombosis, and to reduce the size and alter the composition of the thrombi that are formed (38). Furthermore defibrinogen has a protective effect in animal models on myocardial, liver, and kidney ischemia (39). When healthy volunteers were treated with oral dosages of 5, 10, and 20 mg/kg defibrinogen, a dose-dependent increase in the release of the tissue factor pathway inhibitor (TFPI) was observed (Fig. 14-3). This suggests that, similar to heparin, defibrinogen is capable of releasing TFPI.

Defibrinogen has been demonstrated to be more effective than placebo for the prevention of postoperative deep vein thrombosis but does not appear to be superior to subcutaneous unfractionated heparin (35,40).

TISSUE FACTOR PATHWAY INHIBITOR

Coagulation at a site of injury is initiated by exposure of blood to cell surface tissue

factor and formation of the tissue factor-factor VIIa complex. The latter then activates both factors IX and X, leading to thrombin generation and fibrin formation. Tissue factor pathway inhibitor (TFPI) plays a primary role in regulating tissue factor-induced coagulation. Human plasma contains a tissue factor pathway inhibitor, formerly termed anticonvertin (41), external pathway inhibitor (42), or lipoprotein-associated coagulation inhibitor (LACI) (43).

Tissue factor (TF) is an integral membrane protein of the vascular endothelium, primarily synthesized by the endothelium, that functions as an essential cofactor for the proteolytic activity of factor VII toward its substrates, factors IX and X (44,45).

This endogenous protease inhibitor of 42,000 Da is a 276-amino acid polypeptide consisting of three Kunitz-type serine protease inhibitor domains (Fig. 14-4). The majority (85%) of human TFPI remains associated with apolipoprotein AII, possibly via a mixed disulfide linkage. TFPI interacts with the target proteins of factors VIIa and Xa in a

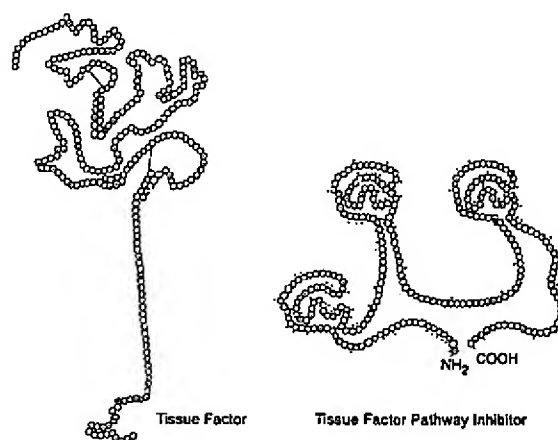


FIG. 14-4. A comparison of the primary structure of tissue factor (TF) with tissue factor pathway inhibitor (TFPI). TF is a transmembrane protein with both plasmatic and TF activating properties. TFPI is capable of selectively inhibiting TF and its mediated responses. Thus, in addition to controlling coagulation activation, this inhibition is also involved in the control of inflammatory responses. (From Fareed et al., ref. 108, with permission.)

two-step fashion. In the first step, which is independent of calcium, TFPI binds to factor Xa by the second Kunitz domain, presumably through an active arginyl site (Fig. 14-5). This reaction does not involve the Glu residues on factor Xa. The bimolecule then induces a feedback inhibition of tissue factor–factor VIIa complex, thereby inhibiting the extrinsic pathway of coagulation. In this second step, the binding occurs through the first Kunitz domain. This reaction requires calcium and the Glu residues on factor Xa are essential (43). The function of the third Kunitz-like domain is unknown but a segment of it, including the C-terminal tail, may be involved in binding to cell surface glycosaminoglycans (46). TFPI may also inhibit factor VIIa–tissue factor complexes in the absence of factor Xa (47). This may be an additional mechanism of action when recombinant TFPI is considered for use as a therapeutic agent (48). Thus TFPI

inhibits factor Xa directly, whereas inhibition of factor VIIa requires the simultaneous presence of factor Xa to form a quaternary complex TFPI–factor Xa and VIIa–tissue factor.

There is accumulating evidence that the tissue factor–induced coagulation system is involved in arterial thrombosis and atherosclerosis. Atherosclerotic plaques contain tissue factor–synthesizing cells, and plaque rupture leads to exposure of tissue factor activity to the circulating blood (49–54). Elevated TF activity has been demonstrated in patients with myocardial infarction (54), even in young age (56), suggesting that TFPI adapts to changes in the activity of factor

Recombinant TFPI is available either full length (prepared from Chinese hamster ovary cells) (44) or in a truncated form (TFPI₁₋₁₆₁) lacking the basic C-terminal region and the third Kunitz domain and is produced in yeast cells (57). The two forms

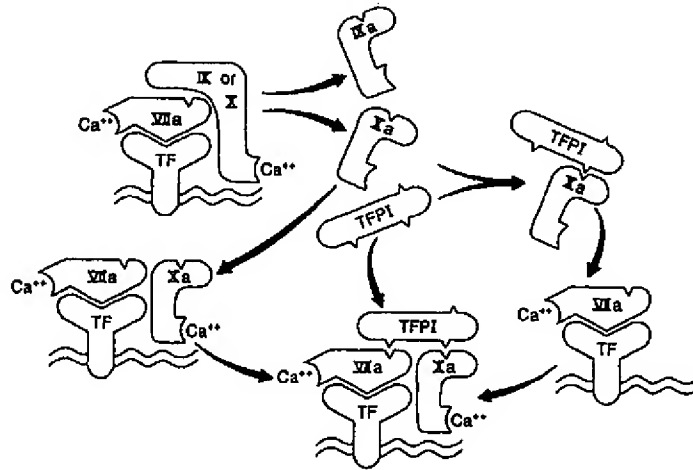


FIG. 14-5. Proposed mechanism for the inhibition of factor Xa and the factor VIIa–tissue factor complex by tissue factor plasminogen inhibitor (TFPI). The indentations represent the active site of factor VIIa and factor Xa; the protrusions represent the three Kunitz-type domains of TFPI. In the final quaternary factor Xa–TFPI–factor VIIa/tissue factor complex, factor Xa is bound at its active site to the second Kunitz domain of TFPI and factor VIIa is bound at its active site to the first Kunitz domain of TFPI. Two potential pathways for the formation of the final quaternary inhibitory complex are depicted: on the left, TFPI binds to a preformed factor Xa–factor VIIa/tissue factor complex; on the right, TFPI binds to factor Xa and the factor Xa–TFPI complex then binds to factor VIIa/tissue factor. (Broze, ref. 109, with permission.)

rTFPI have different pharmacokinetic and activity profiles. Full-length rTFPI binds to factor Xa at a much faster rate; it also binds to endothelial glycosaminoglycans in microvessels in contrast to the two-domain rTFPI (58). Full-length rTFPI is also cleared more rapidly from circulation. The truncated form of TFPI, in contrast to full-length TFPI, does not bind to heparin but has dose-related antithrombotic activity in experimental venous thrombosis in rabbits induced by a combination of endothelium destruction and restricted blood flow (59). Although TFPI₁₋₁₆₁ displayed a dose-dependent increase in activity in the anti-factor Xa, APTT, and PT assays, APTT and PT were, for the same antithrombotic effect, much less prolonged compared with low molecular weight heparin. TFPI has no direct effect on thrombin and does not prolong the clotting time in the anti-factor IIa assay, even at high dose. No bleeding was observed in rabbits or rats receiving 10 mg/kg TFPI₁₋₁₆₁/day, an antithrombotic dose as effective as 60 anti-factor Xa IU/kg of tinzaparin sodium (60). Full-length recombinant TFPI expressed in *E. coli* (61,62) appears to be a much more potent antithrombotic. It completely prevented arterial reocclusion after vessel wall injury that yielded platelet-rich thrombi (63,64) and reduced coagulopathic and lethal effects in the baboon gram-negative model of septic shock (65). The same compound administered for 3 days in a rabbit atherosclerosis injury model reduced angiographic restenosis and decreased neointimal hyperplasia compared with controls (66). This indicates the importance of early inhibitors of the extrinsic pathway in response to arterial injury. Brief inhibition of the coagulation system by administration of TFPI sustains patency of arteries recanalized by pharmacologic fibrinolysis in the dog without markedly perturbing the coagulation system (67).

The hookworm, *Ancylostoma caninum*, produces TFPI which has been characterized and cloned. This small recombinant protein (9500 Da), administered subcutaneously or intravenously, reduces in a dose-dependent

manner coronary and carotid artery occlusions in rat and pig models and in a chronic model of deep venous thrombosis in rats (68). A nematode low molecular weight protein has similar properties.

Administration of recombinant TFPI may become an interesting antithrombotic drug targeted to exposed subendothelium. Full-length rTFPI is presently being used in clinical trials in patients with sepsis and in those with microvascular surgery (7).

INACTIVATED TISSUE FACTOR

Tissue factor has a dominant role in the thrombus formation in arterial wall damage or ruptured atherosclerotic plaques. One may use inactivated factor VIIa (factor VIIai) as a competitive inhibitor of tissue factor-dependent activation of factor X.

Vascular thrombosis at sites of carotid endarterectomy in baboons was prevented by bolus intravenous administration of 1 mg/kg factor VIIai (31,66,69,70). In these experiments hemostasis was not compromised and the bleeding time remained normal. Monoclonal antibodies against tissue factor or against factor VII can also be considered. Synthetic peptides based on nematode-derived peptide antagonists of FVIIa/FVII have also been investigated (68,71).

BLOCKERS OF THE THROMBIN RECEPTOR FUNCTION

There are two thrombin receptors on the platelet membrane that undergo activation by thrombin-mediated cleavage of the terminal peptides yielding neoamino terminal peptide sequences that activate the receptors as tethered ligands (72). Protease-activated receptors 1 and 3 (PAR-1, PAR-3) are reactivated by thrombin and PAR-2 by trypsin (73). Monoclonal antibodies against human PAR-1 and PAR-3 raised in rabbits were tested in nonhuman primates and found to be antithrombotic (74). This approach is attractive as it produces dose-dependent interruption of platelet thrombosis while sparing cleavage of

fibrinogen for the formation of hemostatic plugs (70).

HUMAN PLASMA-DERIVED AND RECOMBINANT ANTITHROMBIN III

The natural plasma protein antithrombin III is a relatively poor inhibitor of thrombin, but its inhibitory effect is increased 10,000-fold in the presence of heparin. In vivo the interaction between antithrombin and heparin probably takes place at the endothelial cell surface where the protein binds to the heparin-like glycosaminoglycan, heparan sulfate. It has been shown that for tight binding of heparin to antithrombin III a particular pentasaccharide sequence must be present (75). This pentasaccharide brings about a change in conformation in antithrombin, which involves an arginine residue that is sufficient for near-maximal acceleration of the inhibition of factor Xa (76). In contrast, much longer heparin species are required for significant acceleration of the rate of inhibition of thrombin by antithrombin III because this reaction appears to be accelerated principally by simultaneous binding of both thrombin and antithrombin III to the same heparin molecule, increasing the inhibition rate of thrombin by antithrombin III encounter frequency (77).

Concentrates of human plasma-derived antithrombin III are available. As with all plasma products, the potential for viral infection must be weighed against the therapeutic benefits and alternatives. The concentrates contain some nonfunctioning, cross-reacting material and so the effect of therapy should be monitored by functional rather than antigenic antithrombin assays (77). The biological half-life of antithrombin from concentrates ranges from a mean of 61 hours (78) to 92 hours (79). The half-life is not affected by the presence of coumarins (74) but is shortened by heparin (80) and in the postoperative period (77).

Because of the moderate survival time of antithrombin III in the circulation, alternate-day substitution is usually sufficient, although activity should be monitored regularly; it seems reasonable to maintain activity above

80% of the normal blood level during treatment period.

In septicemia, where there is widespread endothelial damage, the action of antithrombin III may be impaired. In addition, under conditions of a fulminant inflammatory response, as occurs during *E. coli* sepsis, the expression of heparin-like receptors on the vascular endothelium may be downregulated in the same manner as the thrombomodulin and protein S receptors. This may in part explain why high concentrations of antithrombin III are necessary to prevent shock in animal models of sepsis (81,82). Theoretically, combined antithrombin and heparin therapy should be more effective than antithrombin alone in the management of shock, but unfortunately this form of treatment does not improve the outcome in shocked patients and was associated with an increased risk of bleeding.

In humans, two randomized trials compared antithrombin III with a synthetic protease inhibitor (83) or antithrombin III with heparin (84). Both studies documented a significant attenuation of disseminated intravascular coagulation (DIC) after antithrombin III treatment, but neither included a placebo control group. A placebo-controlled, double-blind trial in patients with septic shock and DIC treatment with a plasma concentrate of antithrombin III achieved significant earlier correction of DIC but failed to decrease mortality in a significant manner (85).

Compared with heparin alone, adjunct intracoronary therapy with a plasma antithrombin III concentrate does not appear to have any beneficial effect on procedural outcome as well as type and frequency of complications during percutaneous coronary angioplasty (PTCA), even in subgroups of patients with a high risk for thrombotic complications (86). Thus, a local deficiency of antithrombin III does not seem to play a major role for the failure of heparin to abolish thrombotic complications during PTCA. It should be noted that antithrombin III in combination with heparin inhibits coronary restenosis in atherosclerotic swine subjected to balloon overstretch (87). A similar study

has not been conducted in patients. In a critical authoritative review of the clinical use of antithrombin III concentrates it was concluded that antithrombin III is only beneficial in a few clinical situations in patients with hereditary antithrombin III deficiency (such as delivery, acute thromboembolic complications, and postoperative prophylaxis). In acquired antithrombin III deficiency, there is no proven indication for the use of antithrombin III concentrates (88).

Different recombinant antithrombin III molecules are now available. Numerous problems had to be solved due to the failure to obtain satisfactory expression of functional or reactivatable antithrombin III from *E. coli* (89). Even expression in yeast gave only poor levels of active antithrombin (90). The majority of reports on active wild-type and variant antithrombin III have thus involved mammalian cell or baculovirus expression. In addition to the greater time involved in obtaining protein from such a system compared to *E. coli*, there is the further problem of heterogeneous glycosylation, which would be absent in bacterial expression. Differences in glycosylation of antithrombin have been shown to affect the affinity of the antithrombin for heparin (91-93). No randomized controlled trials with recombinant antithrombin III were published.

INHIBITORS OF FACTOR VIII

A synthetic 12-amino-acid peptide corresponding to a light-chain residue of factor VIII inhibits cleavage by thrombin of the heavy chain required for the activation of the procoagulant activity of factor VIII and also of the light chain required to dissociate factor VIII from von Willebrand factor. Tyrosine sulfation of the peptide potentiates its recognition by factor VIII (94).

Several other glycosaminoglycans (such as suleparioide, hemoclar, arteparon) and dermatan sulfates (such as heparin sulfate) are being developed, but clinical experience with these as antithrombotic drugs is limited. The same holds true for synthetic hypersulfated bis-lactobionic acid amide (aprosulfate).

NAROPARCIL

It is known that the majority of mammalian tissues produce chondroitin sulfate and heparin sulfate glycosaminoglycan chains when provided with exogenous β -D-xylasides. This is considered to be achieved by competition between the exogenous β -D-xylaside and the xylosylated serines on the core proteins of the endogenous proteoglycans undergoing biosynthesis (95). Naroparcil is a β -D-xylaside (4-[4-cyanobenzoyl]-phenyl)-1,5-dithio- β -D-xylopyranoside (96,97).

Naroparcil reduced thrombus weight in a Wessler stasis model of jugular vein thrombosis in rabbits in a dose-related manner giving an ED₅₀ of 21.9 mg/kg and 36.0 mg/kg after intravenous and oral administration, respectively (97). Venous antithrombotic activity was maximal 2 to 3 hours after intravenous administration and 4 to 8 hours after oral administration. This suggests that naroparcil is not acting directly and that further metabolism is required, even after intravenous administration; this hypothesis is in line with the absence of any *in vitro* anticoagulant effect of the compound.

The antithrombotic effect of naroparcil occurs without increases in either APTT or thrombin time and without detectable anti-factor Xa or antithrombin activity, although a dose-dependent reduction in thrombin generation (only 50% when the antithrombotic effect was maximal) and an increase in plasma dermatan sulfate-like material was observed. At antithrombotic doses no significant effect of naroparcil on bleeding time was noted.

These interesting findings of naroparcil on a venous thrombosis model in the rabbit have to be confirmed in venous and arterial thrombosis models in other animals. It is interesting that the bioavailability following oral administration of naroparcil in rabbits is high.

COAGULATION INHIBITORS DERIVED FROM APROTININ

Recently, a series of potential coagulation inhibitors derived from the bovine pancreatic trypsin inhibitor aprotinin was described.

These aprotinin-derived analogues showed significantly increased inhibition activity toward factor X, factor VIIa-tissue factor complex, factor XIa, and plasma kallikrein (98). In flow chamber experiments with human blood, these compounds significantly inhibited fibrin formation and platelet deposition on extracellular matrix from phorbol ester stimulated human endothelial cells under both high- and low-shear stress and in the presence of low molecular weight heparin (99).

Platelet-dependent thrombus deposition was quantified by dedicated image analysis after transillumination of the hamster femoral vein to which a standardized vascular trauma was applied. All tested aprotinin-derived agents, except for aprotinin, induced a dose-dependent decrease of thrombus formation and concomitant prolongation of the APTT with a complete inhibition of thrombus formation at two- to threefold prolongation of the APTT (100).

These results are to be confirmed in other animal species and the safety profile is to be established before these compounds can be considered as potential candidates for antithrombotic therapy.

INHIBITORS OF FACTOR XIII

In blood, factor XIII is present in two forms. Plasma factor XIII is produced in the liver and consists of a heterotetramer with two catalytic A subunits and two noncatalytic B units (A_2B_2) (101). Platelet factor XIII consists of only two A subunits (102). Both plasma and platelet forms of factor XIIIa contribute to fibrin cross-linking and to fibrin α_2 -antiplasmin ligation in plasma (103). Factor XIIIa also cross-links other plasma proteins to fibrin, such as α_2 -antiplasmin (103) and fibronectin (104); cross-linked fibrin is largely resistant to thrombolytics. This accounts for the interest in finding agents that inhibit the activation of factor XIII or impede its action. Monoclonal antibodies inhibit both plasma and platelet factor XIII (101,104) and a thiadiazole inhibitor (L-722,151) was used in vivo as an antithrombotic agent in thrombolysis experiments (105,106).

CONCLUSION: THE IDEAL ANTITHROMBOTIC DRUG

It is obvious that commercial antithrombotic drugs currently available for clinical use all have serious drawbacks. Moreover, the drugs applied to monitor unfractionated heparin (APTT) and vitamin K antagonist (PT) also have serious shortcomings.

The combination of very-low-dose oral anticoagulants (targeted INR-2) with a potent antiaggregation may be a welcome way out for the prevention of thromboembolic complications in patients at low or medium-thrombotic risk. Future drugs or drug combinations will all have to be tailored in accordance with the pathogenesis of the thrombus (venous versus arterial thrombus, thrombus induced by mechanical trauma as in PTE, thrombus on foreign surfaces as on stents, platelet-rich versus platelet-poor thrombus). Local delivery of antithrombotic drugs or getting to the thrombus may increase the local concentration where most needed and allow lower systemic levels, thereby reducing bleeding risk. With recombinant technology, human natural anticoagulants can be obtained in liberal quantities (antithrombin III, thrombomodulin, protein C, TFPI) and allow the creation of mutants and hybrids.

Table 14-1 summarizes what the ideal antithrombotic drug should be. It is hoped that the properties of one of the numerous antithrombotic drugs in development would correspond to this hematologist's dream of obtaining an antithrombotic drug without anticoagulant properties.

TABLE 14-1. *Ideal antithrombotic agent*

Oral as well as parenteral effectiveness
Rapid onset of action, <1 hour
Rapid cessation of effect by nontoxic antidote
Satisfactory therapeutic index, absence of side effects
No cumulative action or toxicity from long-term use
Predictable quantitative relation between dose and anticoagulant action
Antithrombotic effect not requiring laboratory monitoring
No or limited interaction with commonly used drugs
Large benefit-to-risk ratio
Inexpensive

Thrombolytic Agents

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University of Leuven, B-3000 Leuven, Belgium*

Fibrin-Specific Thrombolytic Agents 303	Non-Fibrin-Specific Thrombolytic Agents 312
Wild-type Tissue-type Plasminogen Activator 303	Two-Chain Urokinase-type Plasminogen
Mutants and Variants of Tissue-type Plasminogen	Activator 312
Activator 305	Streptokinase 313
Single-Chain Urokinase-type Plasminogen	Anisoylated Plasminogen Streptokinase Activator
Activator 307	Complex 314
Staphylokinase 309	Conclusion 315

Cardiovascular diseases, comprising acute myocardial infarction, stroke, and venous thromboembolism, have as their immediate underlying cause thrombosis of critically situated blood vessels with loss of blood flow to vital organs. One approach to the treatment of thrombosis consists of infusing thrombolytic agents to dissolve the blood clot and to restore tissue perfusion and oxygenation. Thrombolytic agents are plasminogen activators which activate the blood fibrinolytic system by activation of the proenzyme, plasminogen, to the active enzyme plasmin. Plasmin in turn digests fibrin to soluble degradation products. Inhibition of the fibrinolytic system occurs both at the level of the plasminogen activators, by plasminogen activator inhibitors (PAI-1 and PAI-2), and at the level of plasmin, mainly by α_2 -antiplasmin. Currently, five thrombolytic agents are available for clinical use: streptokinase, anisoylated plasminogen streptokinase activator complex (APSAC), two-chain urokinase-type plasminogen activator (tcu-PA), single-chain u-PA (scu-PA, prourokinase), and tissue-type plasminogen activator (t-PA).

Streptokinase, APSAC, and tcu-PA induce extensive systemic plasmin generation; α_2 -antiplasmin inhibits circulating plasmin but may become exhausted during thrombolytic therapy because its plasma concentration is only about half that of plasminogen. As a result, plasmin, which has a broad substrate specificity, will degrade several plasma proteins, such as fibrinogen, coagulation factors V, VIII, and XII, and von Willebrand factor. These thrombolytic agents are therefore considered to be non-fibrin-specific. In contrast, the physiologic plasminogen activators, t-PA and scu-PA, are more fibrin-specific because they activate plasminogen preferentially at the fibrin surface and less in the circulation. Plasmin, associated with the fibrin surface, is protected from rapid inhibition by α_2 -antiplasmin because its lysine binding sites are not available and may thus efficiently degrade the fibrin of a thrombus (1,2). The molecular interactions determining the fibrin specificity of plasminogen activators are schematically illustrated in Fig. 17-1.

In patients with acute myocardial infarction, reduction of infarct size, preservation of

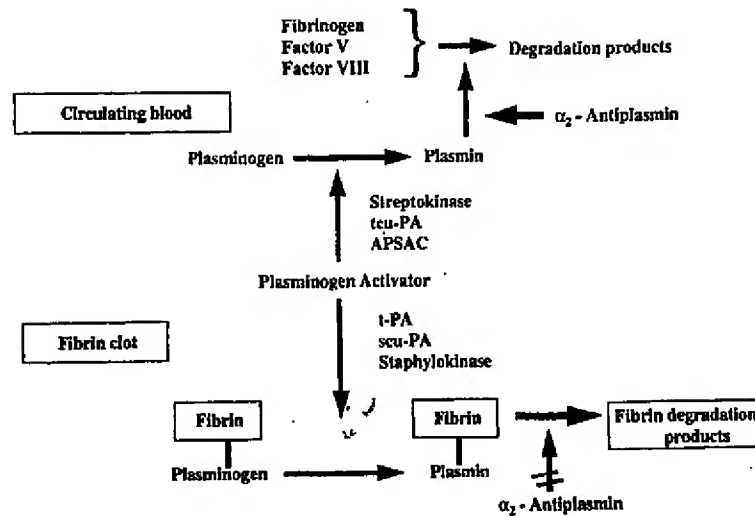


FIG. 17-1. Molecular interactions determining the fibrin specificity of thrombolytic agents. Non-fibrin-specific agents (streptokinase, scu-PA, APSAC) extensively activate plasminogen in the circulating blood, whereas fibrin-specific agents (t-PA, scu-PA, staphylokinase) preferentially activate fibrin-associated plasminogen.

ventricular function, and reduction in mortality has been demonstrated with streptokinase, recombinant t-PA (rt-PA), and APSAC. The GUSTO (Global Utilization of Streptokinase and t-PA for Occluded Coronary Arteries) trial has conclusively established a correlation between early coronary patency and reduction in mortality (3).

Nevertheless, all available thrombolytic agents suffer from significant shortcomings, including large therapeutic doses, short plasma half-lives, limited efficacy and fibrin specificity, reocclusion, and bleeding complications (1,2,4). At best, a brisk coronary flow (TIMI 3 grade) within 90 min is obtained in approximately half of the myocardial infarction patients treated with thrombolytic drugs. To obtain this antegrade coronary flow requires an average of 45 minutes, and reocclusion occurs in roughly 10% of patients. In most trials the residual mortality in patients treated with thrombolytic drugs is not less than half of that without thrombolytic treatment. In the treatment of

myocardial infarction and ischemic stroke, a second limitation is the length of treatment; earlier and accelerated administration of thrombolytic drugs should be a first and immediately applicable goal. If recanalization of an occluded artery must reflect not only the velocity of plasmin activation in the thrombus, but also the rate with which the thrombolytic agent reaches and diffuses into the thrombus, and the rate with which the thrombolytic therapy can be initiated. These ideas have encouraged development of "front-loaded" or "bolus" administration schedules. In the early days of thrombolytic therapy, a rapid course of the thrombolytic effect was thought an advantage, in that thrombolysis could be interrupted rapidly if complications occurred. This has proved more difficult to achieve than anticipated because plasminogen activation may continue within a thrombus after systemic thrombolytic activity has returned to normal. Conversely, agent-induced rapid termination of action may be useful

factory because it might be difficult to ensure completion of clot lysis without the technical difficulties of a carefully controlled infusion. Another limitation is the lack of significant impact of aspirin and heparin on the speed of thrombolysis or resistance to lysis and, importantly, the fact that they do not consistently prevent reocclusion. Paradoxically, fibrinolytic drugs themselves can exert procoagulant effects through generation of plasmin, which may activate the coagulation system resulting in a retardation of apparent thrombolysis, failure of initial recanalization, and virtually instantaneous reocclusion. Thus, vigorous, concomitant anticoagulation is needed. Specific reduction of platelet aggregation is presently being explored with monoclonal antibodies or synthetic peptides against the platelet receptor GPIIb/IIIa. Another approach is the use of selective inhibitors of thrombin, of factor Xa, or of factor VIIa. Furthermore, it is possible that the desired profile for a thrombolytic agent to be used in patients with an acute coronary or carotid-vertebral occlusion, with its emphasis on speed, may not be optimal for agents to be used for other indications, such as venous occlusion.

Therefore the search continues for better thrombolytic agents or regimen. Recent approaches to improve the thrombolytic properties of plasminogen activators include the production of mutant plasminogen activators, of chimeric molecules comprising portions of different plasminogen activators, of antibody-targeted plasminogen activators using fibrin-specific or platelet-specific monoclonal antibodies, and of plasminogen activators from animal or bacterial sources (5-7). Some of these new thrombolytic agents have shown promise in animal models of venous or arterial thrombosis and in pilot clinical studies (7). In this chapter, we will focus on the biochemistry, mechanism of action, and pharmacodynamic properties of presently available thrombolytic agents and of some new and promising agents that are being developed for clinical use.

FIBRIN-SPECIFIC THROMBOLYTIC AGENTS

Wild-type Tissue-type Plasminogen Activator

Physicochemical Properties

The cDNA of t-PA has been cloned and the complete amino acid sequence has been determined (8). The human t-PA gene, localized on chromosome 8 (bands 8.p.12 → q.11.2) consists of 14 exons, and the intron-exon organization suggests that the assembly occurred according to the "exon shuffling" principle, whereby the distinct structural domains are encoded by a single exon or by adjacent exons (for references, cf. 9). The proximal promoter sequences in the human t-PA gene contain typical TATA and CAAT boxes, and potential recognition sequences for several transcription factors have been identified (10,11). Consensus sequences of a cAMP-responsive element and of an AP-2 binding site have been identified, which may have a cooperative effect on constitutive t-PA gene expression (12).

Human t-PA was first isolated as a single-chain serine proteinase of 70 kDa, consisting of 527 amino acids with Ser as the NH₂-terminal amino acid (8); it was subsequently shown that native t-PA contains an NH₂-terminal extension of three amino acids, but in general the initial numbering system has been maintained (Fig. 17-2). Limited plasminic hydrolysis of the Arg²⁷⁵-Ile²⁷⁶ peptide bond converts t-PA to a two-chain molecule held together by one interchain disulfide bond. The t-PA molecule contains four domains: (a) an NH₂-terminal region of 47 residues (residues 4 to 50) (F domain), which is homologous with the finger domains mediating the fibrin affinity of fibronectin; (b) residues 50 to 87 (E domain), which are homologous with epidermal growth factor; (c) two regions comprising residues 87 to 176 and 176 to 262 (K₁ and K₂ domains), which share a high degree of homology with the five kringles of plasminogen; and (d) a serine proteinase domain

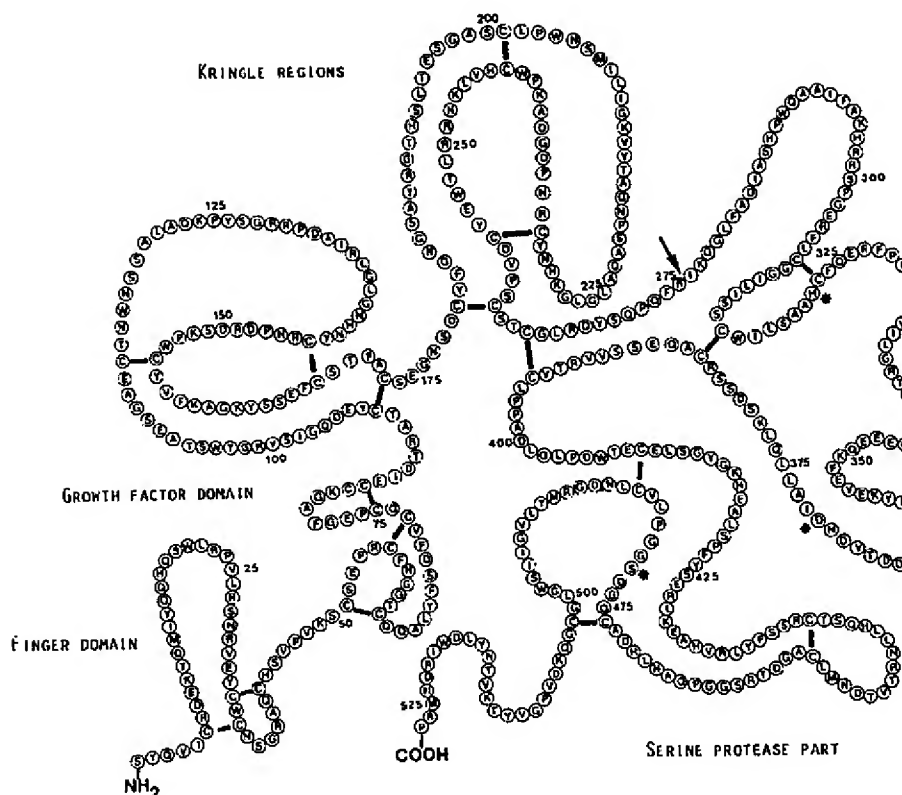


FIG. 17-2. Schematic representation of the primary structure of rt-PA. The amino acids are represented by their single-letter symbols and black bars indicate disulfide bonds. The active site residues are indicated with an asterisk. The arrow indicates the plasmin cleavage site for conversion of single-chain rt-PA to two-chain rt-PA. In reteplase (BM 06.022), the sequence comprising Val⁴ thru Glu¹⁷⁸ has been deleted. In TNK-rt-PA, Thr¹⁰³ is substituted by Asn, Asn¹¹⁷ by Gln, and the sequence Lys²⁸⁶-His-Arg-Arg²⁸⁹ is mutagenized to Ala-Ala-Ala-Ala.

(P, residues 276 to 527) with the active site residues His³²², Asp³⁷¹, and Ser⁴⁷⁸. There are three potential N-glycosylation sites, at Asn¹¹⁷ (K₁), Asn¹⁸⁴ (K₂), and Asn⁴⁴⁸ (P). t-PA preparations usually contain a mixture of variant I (with all three glycosylation sites) and variant II (lacking carbohydrate at Asn¹⁸⁴) (8). The t-PA molecule is ellipsoidal and relatively compact (13), with the individual domains folded within the molecule yielding a globular structure, which is stabilized by strong interactions between the proteinase domain and the F and/or E domains (14,15). In contrast to the single-chain precursor form of most serine

proteinases, single-chain t-PA is enzymically active. On the basis of conformational similarities between single-chain and chain t-PA, it was postulated that the activation of single-chain t-PA would involve an equilibrium between an active and a zymogenic form, which would be shifted to the active conformation upon substrate binding (16). Amino acid Lys¹⁵⁶ appears to play a role in the enzymatic activity of single-chain t-PA by stabilizing the active conformation (17).

Inhibition of t-PA by its physiological inhibitor PAI-1 involves formation of a 1:1 stoichiometric reversible complex, followed

covalent binding between the hydroxyl group of the active site Ser⁴⁷⁸ residue of t-PA and the carboxyl group of the P₁ residue at the reactive center (Arg³⁴⁶) of the inhibitor. The reversible high-affinity second-site interaction occurs between a negatively charged sequence in PAI-1 (residues 350 to 355) and a positively charged region in t-PA (residues 296 to 304) (for references, cf. 9).

Mechanism of Action

As all known plasminogen activators, t-PA converts its substrate plasminogen to plasmin by cleavage of a single Arg⁵⁶¹-Val⁵⁶² peptide bond. t-PA is a poor enzyme in the absence of fibrin, but the presence of fibrin strikingly enhances the activation rate of plasminogen. Optimal stimulation is only obtained after early plasmin cleavage in the COOH-terminal α chain and the NH₂-terminal β chain of fibrin, yielding fragment X polymer (18). Kinetic data (19) support a mechanism in which fibrin provides a surface to which t-PA and plasminogen adsorb in a sequential and ordered way yielding a cyclic ternary complex. Formation of this complex allows an enhanced affinity of t-PA for plasminogen, resulting in an up to three orders of magnitude higher catalytic efficiency for plasminogen activation. Plasmin formed on the fibrin surface has both its lysine binding sites and active site occupied and is thus only slowly inactivated by α_2 -antiplasmin; in contrast, free plasmin, when formed, is very rapidly inhibited by α_2 -antiplasmin (for references, cf. 9).

Pharmacokinetic Properties

rt-PA (predominantly two-chain material) is cleared from the circulation in a biphasic manner with an initial half-life of 6 minutes and a terminal half-life of 64 minutes in humans (20). The initial and terminal half-lives of single-chain rt-PA, following infusion of 8.3 μ g per kg per minute over 30 minutes in healthy volunteers, were 3.3 and 26 minutes (21). In patients with acute myocardial infarction, clearance of single-chain rt-PA was found to be

30% to 40% more rapid than two-chain rt-PA (22). Clearance is the result of interaction with several receptor systems. Liver endothelial cells have a mannose receptor that recognizes the high-mannose-type carbohydrate side chain at Asn¹¹⁷ in the K₁ domain, whereas liver parenchymal cells contain a calcium-dependent receptor that interacts mainly with the growth factor domain of t-PA (23,24). In addition, the low-density lipoprotein receptor-related protein (LRP), expressed in high copy number on hepatocytes, binds free t-PA and complexes with PAI-1 (25,26).

Dosage

The recommended dose of recombinant t-PA (alteplase, Activase, Actilyse) for the treatment of acute myocardial infarction was 100 mg administered as 60 mg in the first hour (of which 6 to 10 mg is administered as a bolus over the first 1 to 2 minutes), 20 mg over the second hour, and 20 mg over the third hour. More recently, it was proposed to give the same total dose of 100 mg but "front-loaded," starting with a bolus of 15 mg followed by 50 mg in the next 30 minutes and the remaining 35 mg in the following hour (27). In the GUSTO trial, a dose of 15 mg intravenous bolus of alteplase followed by 0.75 mg per kg over 30 minutes (not to exceed 50 mg) and then 0.50 mg per kg over 60 minutes (not to exceed 35 mg) was utilized (3). In the COBALT trial, double-bolus administration of rt-PA (50 mg given 30 minutes apart) was evaluated in patients with myocardial infarction (28). Whichever regimen is used, it is important to coadminister intravenous heparin during and after alteplase treatment. For catheter-directed local thrombolysis with alteplase in patients with recent peripheral arterial occlusion, a dose of 0.05 to 0.10 mg per kg per hour over an 8-hour period is usually recommended.

Mutants and Variants of Tissue-type Plasminogen Activator

By deletion or substitution of functional domains, by site-specific point mutations,

and/or by altering the carbohydrate composition, mutants of rt-PA have been produced with higher fibrin specificity, more zymogenicity, slower clearance from the circulation, and resistance to plasma proteinase inhibitors.

During thrombolytic therapy there is a vast excess of t-PA over PAI-1 in the circulation, but critical lysis occurs at the surface of an arterial thrombus where the local PAI-1 concentration can be very high (29). Therefore, mutants with resistance to PAI-1 may be useful to reduce reocclusion. In addition, mutants with prolonged half-life may allow efficient thrombolysis by bolus administration at a reduced dose. Several mutants and variants of t-PA are presently evaluated at the preclinical level in animal models of venous and arterial thrombosis and in pilot studies, mainly in patients with acute myocardial infarction. These agents include reteplase (Rapilysin or Ekokinase), TNK-rt-PA, and the vampire bat (*Desmodus rotundus*) salivary plasminogen activator.

Physicochemical Properties

Reteplase (BM 06.022) is a single-chain nonglycosylated deletion variant consisting only of the kringle 2 and the proteinase domain of human t-PA; it contains amino acids 1–3 and 176–527 of rt-PA (deletion of Val⁴-Glu¹⁷⁵; cf. Fig. 17-2). The Arg²⁷⁵-Ile²⁷⁶ plasmin cleave site is maintained (30).

In TNK-rt-PA, replacement of Asn¹¹⁷ with Gln (N117Q) deletes the glycosylation site in K₁, whereas substitution of Thr¹⁰³ by Asn (T103N) reintroduces a glycosylation site in K₁, but at a different locus; these modifications substantially decrease the plasma clearance rate. In addition, the amino acids Lys²⁹⁶-His²⁹⁷-Arg²⁹⁸-Arg²⁹⁹ were each replaced with Ala (cf. Fig. 17-2) (31).

Different molecular forms of the *Desmodus* salivary plasminogen activator (DSPA) have been purified, characterized, cloned, and expressed. Two high molecular weight forms, DSPA α_1 (43 kDa) and DSPA α_2 (39 kDa), exhibit about 85% homology to human t-PA, but contain neither a kringle 2 domain nor a plas-

min-sensitive cleavage site. DSPA β lacks finger domain and DSPA γ lacks the finger and epidermal growth factor domain (32–34).

Mechanism of Action

The active site in the proteinase domain of reteplase and of t-PA and their plasminogenolytic activity in the absence of stimulator do not differ, but the plasminogenolytic activity of reteplase in presence of CNBr fragments of fibrinogen as a stimulator was found to be fourfold lower compared to t-PA, whereas the binding of reteplase to fibrin was fivefold lower (30). These differences might possibly be due to the missing finger domain in reteplase. It is known that fibrin binding is mediated through both the finger domain and the lysine binding site in the kringle 2 domain of t-PA. Reteplase and t-PA are inhibited by PAI-1 to a similar degree. The affinity of reteplase for binding to endothelial cells and monocytes is reduced compared to t-PA, probably as a consequence of deletion of the finger and epidermal growth factor domains in reteplase which seem to be involved in the interaction with endothelial cell receptors (36).

TNK-rt-PA has a similar ability as wild type rt-PA to bind to fibrin and to lyse fibrin clots in a plasma milieu (31). It has an enhanced fibrin specificity, resistance to inhibition by PAI-1, and slower plasma clearance (37).

DSPA α_1 and DSPA α_2 exhibit a specific activity in vitro that is equal to or higher than that of rt-PA, a relative PAI-1 resistance, a greatly enhanced fibrin specificity without strict requirement for polymeric fibrin as cofactor (32,34).

Pharmacokinetic Properties

Pharmacokinetic analysis of plasma activity in the rabbit revealed a half-life of 18–1.5 minutes for reteplase and 2.1 ± 0.1 minutes for alteplase, with a 4.3-fold slower plasma clearance for reteplase than for

teplase (38). In healthy human volunteers (39) and in patients with acute myocardial infarction (40), an initial half-life of 14 to 18 minutes was observed for reteplase.

TNK-rt-PA has a slower clearance and marked resistance to PAI-1. It was shown to have an increased thrombolytic potency on platelet-rich clots in rabbits, to conserve fibrinogen, and to be effective upon bolus administration at half the dose of rt-PA (31,41). Similar results were obtained in a combined arterial and venous thrombosis model in the dog (42) and in a rabbit carotid artery thrombosis model (43). In patients with acute myocardial infarction, TNK-rt-PA has a plasma clearance of 151 ± 55 ml per minute and a half-life of 17 ± 7 minute, as compared to 572 ± 132 ml per minute and 3.5 ± 1.4 minute for wild-type rt-PA (44).

In several animal models of thrombolysis, DSPA α 1 has a 2.5 times higher potency and four- to eightfold slower clearance than t-PA (45-49). Recombinant DSPA α 1 produced in mammalian cell culture may be suitable for bolus administration, whereby its long half-life and high specific activity may allow a reduction of the therapeutic dose (49).

Dosage

Different doses of reteplase in patients with acute myocardial infarction were evaluated in two open nonrandomized pilot trials (50,51). The randomized RAPID I trial showed that reteplase, when given as a double bolus of 10 plus 10 MU 30 minutes apart, achieves more rapid, complete, and sustained thrombolysis than standard dose alteplase (100 mg over 3 hours) (52). In the RAPID II trial, the same reteplase dose regimen appeared to achieve higher rates of early reperfusion than front-loaded alteplase (53). In the INJECT study, a double-blind randomized trial in patients with acute myocardial infarction, administration of two boluses of 10 MU reteplase given 30 min apart was compared with streptokinase (1.5 MU intravenously over 60 minutes) (54).

In the Thrombolysis in Myocardial Infarction (TIMI) 10A trial, a phase I dose-ranging

pilot study in patients with acute myocardial infarction, single-bolus TNK-rt-PA was administered over 5 to 10 seconds with doses ranging from 5 to 50 mg (44). The agent was fibrin-specific with initial patency and safety profile at 30- to 50-mg doses, which appeared encouraging.

Single-Chain Urokinase-type Plasminogen Activator

Physicochemical Properties

Urokinase (u-PA) was first found in urine at relatively high concentrations (200 to 300 ng/ml) and was later identified in human plasma at a level of about 3 to 5 ng/ml. u-PA is secreted as a single-chain molecule (scu-PA, pro-urokinase) that may be converted to a chain-chain form (tcu-PA). The cDNA has been cloned and expressed (55).

scu-PA is a serine proteinase of 411 amino acids in a single polypeptide chain, with active site triad His²⁰⁴, Asp²⁵⁵, and Ser³⁵⁶ (Fig. 17-3). The molecule contains an NH₂-terminal growth factor domain and one kringle structure homologous to the five kringles found in plasminogen and the two kringles in t-PA (51). u-PA contains only one N-glycosylation site (at Asn³⁰²), and contains a fucosylated threonine residue at position 18. Conversion of scu-PA to tcu-PA occurs after proteolytic cleavage at position Lys¹⁵⁸-Ile¹⁵⁹ by plasmin (56), but also by kallikrein (57), trypsin, cathepsin B, human T-cell-associated serine proteinase-1, and thermolysin. A fully active tcu-PA derivative is obtained after additional proteolysis by plasmin at position Lys¹³⁵-Lys¹³⁶. A low molecular weight form of scu-PA (32 kDa) can be obtained by selective cleavage at position Glu¹⁴³-Leu¹⁴⁴ (58); this cleavage can be obtained with matrix metalloproteinase Pump-1 (59). In contrast, scu-PA is converted to an inactive two-chain molecule by thrombin after proteolytic cleavage at position Arg¹⁵⁶-Phe¹⁵⁷ (57). This inactivation is strongly enhanced in the presence of thrombomodulin and is dependent on the O-linked glucosaminoglycan of thrombomod-

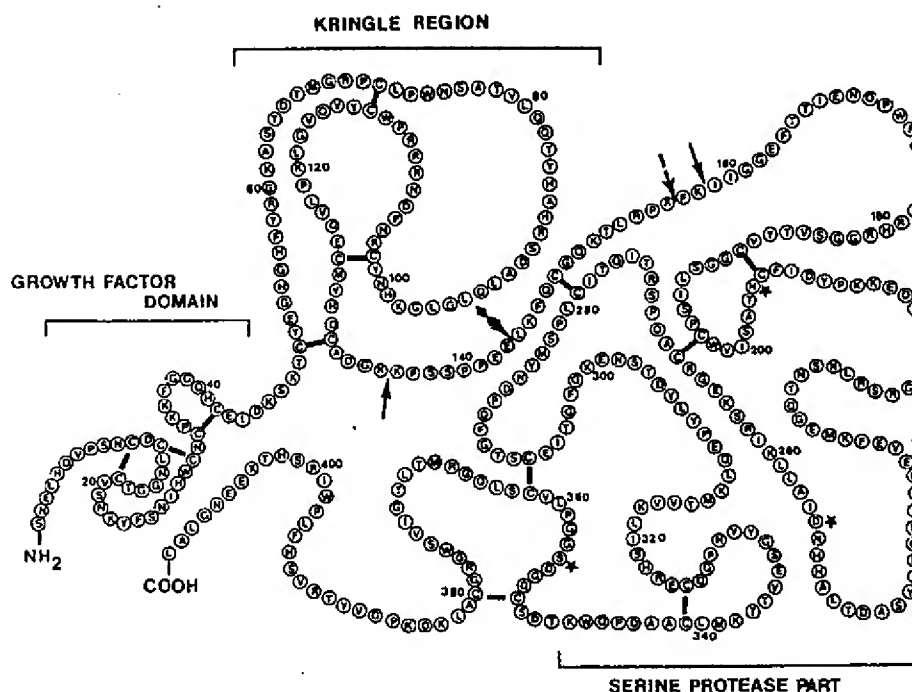


FIG. 17-3. Schematic representation of the primary structure of scu-PA. The amino acids are represented by their single letter symbols and black bars indicate disulfide bonds. The active site residue is indicated with an asterisk. The arrows indicate the plasmin cleavages sites for conversion of 33 kDa scu-PA to 54 kDa tcu-PA (Lys¹⁵⁸-Ile¹⁵⁹), and of 54 kDa tcu-PA to 33 kDa tcu-PA (Lys¹³⁵-Lys¹³⁶), the thrombin cleavage site (Arg¹⁵⁸-Phe¹⁵⁷) yielding inactive 54 kDa tcu-PA, and the conversion to 32 kDa scu-PA (Glu¹⁴³-Leu¹⁴⁴).

ulin (60). The cofactor effect of thrombomodulin on the inactivation of scu-PA by thrombin could be demonstrated in a perfused rabbit heart model (61).

Mechanism of Action

u-PA is a serine proteinase with a high substrate specificity for plasminogen. In contrast to tcu-PA, scu-PA displays very low activity toward low molecular weight chromogenic substrates. Scu-PA appears to have some intrinsic plasminogen activating potential, which represents $\leq 0.5\%$ of the catalytic efficiency of tcu-PA (62,63). However, other investigators have claimed that scu-PA has no measurable intrinsic amidolytic or plasmino-

gen activator activities (64). The occurrence of a transitional state of scu-PA with a high catalytic efficiency for native plasminogen than tcu-PA has been postulated (65). Furthermore, it was reported that fibrin fragment E-2 selectively promotes the activation of plasminogen by scu-PA, mainly by enhancing the catalytic rate constant of the activated complex (66). Indeed scu-PA is not an efficient activator of plasminogen bound to internal lysine residues on intact fibrin, but it develops higher activity toward plasminogen bound to newly generated COOH-terminal lysine residues on partially degraded fibrin (67). Subsequent studies confirmed that the high specificity of scu-PA does not require its conversion to tcu-PA but appears to be medi-

by enhanced binding of plasminogen to partially digested fibrin (68).

In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen; in the presence of a fibrin clot, scu-PA, but not tcn-PA, induces fibrin-specific clot lysis (62). scu-PA does not bind to a significant extent to fibrin, although in the presence of Zn^{2+} ions some binding has been reported (69). The intrinsic activity of scu-PA toward fibrin-bound plasminogen may contribute to its fibrin specificity. Furthermore, α_2 -antiplasmin in plasma prevents conversion of scu-PA to tcn-PA outside the clot and thus preserves fibrin specificity (70).

Pharmacokinetic Properties

The turnover of different molecular forms of u-PA in blood of different animal species occurs with an initial half-life of approximately 3 to 7 minutes (62,71-74). Because similar turnover rates were observed for ^{125}I -labeled tracer, enzymatic activity, and antigen, this short half-life seems to be an inherent property of u-PA. The main mechanism of removal of u-PA from the blood appears to occur by hepatic clearance. Experimental hepatectomy in rabbits indeed markedly prolongs the initial half-life of scu-PA (from 3 minutes to 20 to 30 minutes) (71). scu-PA is taken up in the liver via a recognition site on parenchymal cells and is subsequently degraded in the lysosomes (75).

The rapid clearance of u-PA apparently is not mediated via carbohydrate receptors because very similar turnover characteristics are observed for both the scu-PA and tcn-PA forms of unglycosylated recombinant molecules are of glycosylated natural molecules. It does not occur via reaction with plasma proteinase inhibitors and subsequent rapid clearance of the complexes because active site blocked tcn-PA and scu-PA, which do not react with plasma protease inhibitors, have the same half-life as the active tcn-PA forms (71). In addition, the similar half-life observed for 32 kDa scu-PA indicates that clearance is not

mediated via the NH_2 -terminal portion of the molecule (72).

Following intravenous infusion of natural or recombinant scu-PA in patients with acute myocardial infarction, a biphasic disappearance was observed with initial half-lives in plasma (postinfusion) of 4 minutes or 8 minutes, respectively (76,77). This short half-life suggests that the maintenance of a therapeutic level of the agent in plasma may require its continuous infusion.

Dosage

Saruplase is the generic name for full-length unglycosylated human recombinant scu-PA obtained from *Escherichia coli*. With a preparation containing 160,000 IU per mg, the dose used successfully in patients with acute myocardial infarction (PRIMI Study) was 20 mg given as a bolus and 60 mg over the next 60 minutes, immediately followed by an intravenous heparin infusion (20 IU per kg per hour) for 72 hours (78). In the LIMITS Study in patients with acute myocardial infarction, the same dose regimen of saruplase was used, but with a prethrombolytic heparin bolus of 5,000 IU and an intravenous heparin infusion for 5 days starting 30 minutes after completion of thrombolysis (79). A recombinant glycosylated form of prourokinase (A-74187) has been evaluated in patients with acute myocardial infarction using 60 or 80 mg monotherapy or 60 mg primed with a preceding bolus of 250,000 IU of recombinant tcn-PA, always combined with aspirin and intravenous heparin (80).

Staphylokinase

Physicochemical Properties

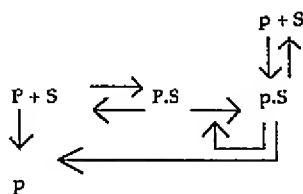
Staphylokinase, a plasminogen activator produced by certain strains of *Staphylococcus aureus*, was shown to have profibrinolytic properties more than four decades ago (81,82). Natural staphylokinase has been purified from *S. aureus* strains that were transformed with bacteriophages containing the

staphylokinase gene or that had undergone lysogenic conversion to staphylokinase production (83,84). In addition, the staphylokinase gene has been cloned from the bacteriophages *sakC* (85) and *sak42D* (86) as well as from the genomic DNA (*sakSTAR*) of a lysogenic *S. aureus* strain (84).

The staphylokinase gene encodes a protein of 163 amino acids with amino acid 28 corresponding to the NH₂-terminal residue of the mature protein, which consists of 136 amino acids in a single polypeptide chain without disulfide bridges. The mature proteins *SakSTAR*, *SakC*, and *Sak42D* differ in only three amino acids; amino acid 34 is Ser in *SakSTAR* but Gly in *SakC* and *Sak42D*; amino acid 36 is Gly in *SakSTAR* and in *SakC*, but Arg in *Sak42D*; and amino acid 43 is His in *SakSTAR* and in *SakC*, but Arg in *Sak42D* (87–89). In purified preparations, different molecular forms of staphylokinase with slightly different molecular weight and isoelectric points have been observed; molecular forms have been characterized lacking the 6 or the 10 NH₂-terminal amino acid (84,88,89).

Mechanism of Action

Staphylokinase forms a 1:1 stoichiometric complex with plasmin(ogen) (90,91). It is not an enzyme, and generation of an active site in its equimolar complex with plasminogen requires conversion of plasminogen to plasmin. Thus, the plasmin–staphylokinase complex is the active enzyme (92,93). This is in contrast with streptokinase, which produces a complex with plasminogen that exposes the active site in the plasminogen moiety without proteolytic cleavage (94). Kinetic data suggest the following mechanism for plasminogen activation in a buffer milieu.



Plasmin (P) and staphylokinase (S) produce an inactive 1:1 stoichiometric complex (P+S) which does not activate plasminogen. The activation reaction appears to be initiated by trace amounts of plasmin (p), which generates an active plasmin–staphylokinase complex (p.S) (93). In mixtures with excess plasminogen over staphylokinase, generated p–S converts excess plasminogen to plasmin. In addition, kinetic analysis has revealed that generated p–S converts P–S to p–S, thus representing a potential positive feedback mechanism (95). However, the main pathway for plasmin generation in the above scheme appears to be activation of P by p–S formed by binding of p to S. This is supported by binding data obtained with fluorescently labeled staphylokinase, showing that it has a much higher affinity for plasmin than for native plasminogen (96).

In a buffer milieu, α_2 -antiplasmin rapidly inhibits the p–S complex if the lysine binding sites in the plasmin moiety of the complex are available (92,97,98). Fibrin, but fibrinogen, reduces the inhibition rate of the complex by α_2 -antiplasmin by competing interaction with the lysine binding site. By delaying inhibition of plasmin or the complex by α_2 -antiplasmin, fibrin thus facilitates generation of active p–S complex (99). However, staphylokinase dissociates from the p–S complex following neutralization by α_2 -antiplasmin and is recycled to other plasmin(ogen) molecules (100). Thus, extensive systemic plasminogen activation with staphylokinase would be expected in plasmin which is in contradiction with its observed fibrin specificity. This may be explained by the finding that in the absence of fibrin, significant amounts of p–S are generated but cause traces of plasmin to be inhibited by α_2 -antiplasmin. In the presence of fibrin, generation of the p–S complex is facilitated because traces of fibrin-bound plasmin are protected from α_2 -antiplasmin and, furthermore, inhibition of p–S by α_2 -antiplasmin at the clot surface is delayed more than 10-fold. Thus, generated p–S may efficiently convert P–S to p–S and excess P to p. R

cling of staphylokinase to fibrin-bound plasmin, after slow neutralization of the p-S complex, will result in more efficient generation of the complex. In addition, binding data obtained with fluorescently labeled staphylokinase indicate that it does not bind to a significant extent to plasminogen in circulating plasma, but binds with high affinity to plasmin and to plasminogen which is bound to partially degraded fibrin (96).

Recently, biochemical studies with highly purified recombinant staphylokinase, initial experiments in animal models of thrombosis, and pilot studies in patients with acute myocardial infarction or peripheral arterial occlusion have revealed that staphylokinase is an efficient and highly fibrin-specific plasminogen activator (101,102).

Pharmacodynamic Properties

In patients with acute myocardial infarction treated with an intravenous infusion of 10 mg staphylokinase (SakSTAR) over 30 minutes, the concentration of staphylokinase-related antigen in blood at the end of the infusion increased to between 0.9 and 1.7 $\mu\text{g/ml}$. The postinfusion disappearance of staphylokinase-related antigen from plasma occurred in a biphasic manner with a $t_{1/2\alpha}$ of 6.3 minutes and a $t_{1/2\beta}$ of 37 minutes, corresponding to a plasma clearance of 270 ml/min (103). In the STAR trial, SakSTAR antigen levels in 25 patients with acute myocardial infarction receiving 10 mg IV over 30 minutes were $0.56 \pm 0.06 \mu\text{g/ml}$ at 25 minutes and $0.16 \pm 0.04 \mu\text{g/ml}$ at 90 minutes, with corresponding levels of $1.9 \pm 0.22 \mu\text{g/ml}$ and $0.42 \pm 0.06 \mu\text{g/ml}$ in 23 patients receiving 20 mg SakSTAR over 30 minutes (104).

Unfortunately, the somewhat low-grade antigenicity of staphylokinase, as suggested by early dog and baboon experiments, is not extended to patients. The vast majority of patients with either myocardial infarction (103,104) or peripheral arterial occlusion (105) developed neutralizing antibodies to SakSTAR, albeit after a long lag phase of 7 to 12 days, that remained elevated well above

pretreatment levels for several months after administration (106). However, the titers of preformed anti-SakSTAR antibodies in the general population appeared to be lower than those of antistreptokinase antibodies (107), and even systemic *S. aureus* infections failed to induce SakSTAR-neutralizing antibodies in most patients (105), possibly reflecting the low proportion of *S. aureus* strains that produce staphylokinase. The boost of neutralizing antibody titers upon infusion of SakSTAR, however, predicts therapeutic refractoriness on repeated administration. Therefore, the restriction to single use applies probably both to streptokinase and staphylokinase. The absence of cross-reactivity to streptokinase of antibodies elicited by SakSTAR, and vice versa, suggests that the consecutive use of both plasminogen activators may be feasible (107). Furthermore, variants of recombinant staphylokinase with reduced immunogenicity have been obtained by site-directed mutagenesis (108–112). Thus, substitution mutagenesis in recombinant staphylokinase (SakSTAR) of clusters of two or three charged amino acids with alanine identified two variants, SakSTAR.M38 (with K35, E38, K74, E75, and R77 substituted with A) and SakSTAR.M89 (with K74, E75, R77, E80, and D82 substituted with A) that had a markedly reduced expression of two of the three immunodominant epitopes of SakSTAR but also had an approximately 50% reduced specific activity. These mutants did not recognize approximately one third of the antibodies elicited in patients by treatment with wild-type SakSTAR, and elicited markedly less circulating neutralizing antibodies and significantly less resistance to thrombolysis in rabbits than wild-type SakSTAR. In patients with peripheral arterial occlusion given doses of 6.5 to 12 mg of compound, SakSTAR.M38 and SakSTAR.M89 induced significantly less neutralizing antibody and staphylokinase-specific IgG than wild-type SakSTAR (109).

In a subsequent study, the effect of the reversal of one or more of these substituted amino acids on the ratio of activity to anti-

genicity was evaluated (111). In pooled plasma from patients with peripheral arterial occlusion treated with wild-type SakSTAR, about 40% of the antibodies depended on K74 of epitope K74, E75, and R77 for binding, whereas epitopes K35, E38 and E80, and D82 had a negligible contribution toward antibody recognition. SakSTAR (K74), with a single substitution of Lys⁷⁴ with Ala, had an intact specific activity but did not absorb 40% of the antibodies induced in patients by treatment with wild-type SakSTAR. The thrombolytic potency and antibody induction of SakSTAR (K74) and of SakSTAR (K74ER) with Lys⁷⁴, Glu⁷⁵, and Arg⁷⁷ replaced by Ala were studied in more detail (112). Intra-arterial administration in patients with peripheral arterial occlusion of SakSTAR (K74) or SakSTAR (K74ER) induced significantly less circulating neutralizing antibody than SakSTAR. Overt neutralizing antibody induction (>10 µg compound neutralized/ml plasma) occurred in all 9 patients given wild-type SakSTAR, in 6 of the 11 SakSTAR (K74) patients, and in 2 of the 6 SakSTAR (K74ER) patients. Thus, SakSTAR (K74) and SakSTAR (K74ER) appear to have intact thrombolytic potencies but induce significantly less antibody formation in patients.

These variants provide proof of concept that reduction of the immunogenicity and immunoreactivity of recombinant staphylokinase by protein engineering may be feasible.

Dosage

In the first pilot recanalization studies, patients with acute myocardial infarction were given 10 mg IV SakSTAR, as a 1-mg bolus followed by infusion of 9 mg over 30 minutes (103). In the STAR trial, patients randomized to intravenous SakSTAR were given 10 mg over 30 minutes in the first half of the study and, following a prospectively planned interim analysis, 20 mg over 30 minutes in the second half, always with an initial 10% bolus (104). In a phased, angiographically controlled pilot study on bolus Sak42D infusion for coronary thrombolysis, 20 mg Sak42D was given over 5

minutes at study entry, with a second bolus 10 mg over 5 minutes at 60 minutes if angiography showed TIMI perfusion grade 0, 1, or 2 (113). The encouraging experience obtained in this pilot study inspired a multicenter randomized trial in patients with evolving myocardial infarction, comparing accelerated rt-PA with double bolus of 15 mg Sak42D given 30 minutes apart.

In a pilot study in patients with peripheral arterial occlusion, intra-arterial catheter-directed SakSTAR was given as a bolus of 1 mg followed by a continuous infusion of 0.5 mg per hour, or as a 2-mg bolus followed by an infusion of 1 mg per hour, together with heparin. Complete recanalization was obtained in 10 of the patients after 7.0 ± 0.7 mg SakSTAR infused over 8.7 ± 1.0 hours (105).

NON-FIBRIN-SPECIFIC THROMBOLYTIC AGENTS

Two-Chain Urokinase-type Plasminogen Activator

Physicochemical Properties

Urokinase is a naturally occurring plasminogen activator excreted in human urine from which it can be extracted; urokinase can also be isolated from tissue cultures of human embryonic kidney cells. It is a trypsin-like enzyme composed of two polypeptide chains: a light chain with 158 amino acids and a heavy chain with 253 amino acids. Urokinase occurs in two molecular forms designated S-PA (54 kDa, low molecular weight u-PA) and Sc-PA (72 kDa, high molecular weight u-PA), the former being a proteolytic degradation product of the latter generated by plasmin cleavage of the Lys¹³⁵-Lys¹³⁶ peptide bond (114). The 54-kDa molecule is generated by proteolytic cleavage of the single-chain precursor PA (cf. supra).

Mechanism of Action

scu-PA activates plasminogen directly following Michaelis-Menten kinetics. The heavy chain molecule has no specific affinity

Exhibit 25

ANTIPLATELET THERAPY IN CLINICAL PRACTICE

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Aspirin and dipyridamole

Freek WA Verheugt

Introduction

Thrombosis plays a major role in the pathogenesis of cardiovascular diseases. Thromboses in both coronary and cerebral arteries are complications of atherosclerosis, the most important single cause of mortality in the Western world. Both myocardial and cerebral infarction cause impressive mortality and morbidity in millions of patients each year. Not only arterial thrombosis but also thrombosis in the heart cavities is responsible for the major morbidity and mortality of cardiovascular disease. Intracardiac thrombosis can be seen in atrial fibrillation, in patients with artificial valves and in those with left ventricular aneurysms.

From the 1950s antithrombotic therapy has been applied with large success to prevent these frequently fatal thrombotic complications. Anticoagulants (heparin and coumadin) were the first antithrombotic agents used in the prevention and treatment of arterial thrombosis. In the 1970s agents interfering with platelet function were introduced and found to be effective in the prevention and treatment of arterial thrombosis. Males and females benefit equally from antiplatelet therapy in the secondary prevention of cardiovascular disease.¹ In hypertensive patients, the efficacy of antiplatelet agents is similar to that in normotensives and in diabetes antiplatelet therapy is as effective as in non-diabetics.¹

The most regularly used antiplatelet agents in cardiovascular disease are aspirin (acetylsalicylic acid) and dipyridamole (Persantin).¹

Pharmacology of aspirin and Persantin

Pharmacology of aspirin

The effect of aspirin on haemostasis has been recognized for several decades. The second step of the aggregation of blood platelets by ADP is almost completely blocked by the drug.

Platelet aggregation is one of the pathways through which clots are formed on damaged arterial wall. Platelets can aggregate under several stimuli, one of which is mediated through thromboxane A₂, a platelet specific prostaglandin that can only be generated in the presence of cyclo-oxygenase. Cyclo-oxygenase production is regulated in the cell nucleus. Aspirin inactivates cyclo-oxygenase. Since platelets lack nuclei, cyclo-oxygenase is inactivated irreversibly by aspirin during the life of the platelet. Therefore, one dose of aspirin blocks, almost completely, and for several days the platelet's ability to aggregate via the thromboxane pathway and produce further thromboxane (Figure 6.1). Thromboxane promotes platelet aggregation and also vasoconstriction. Each active mechanism in nature is counterbalanced: in this case, platelet

75-100 mg three times daily. Recently a slow-release formulation of 200 mg to be given twice daily has become available.

Side-effects of dipyridamole

Owing to its vasodilating properties, dipyridamole may induce headache and vertigo; sometimes flushing is noted. Nausea, vomiting and diarrhoea are rare side-effects. These adverse effects usually disappear rapidly following drug withdrawal.

Conclusions

Both aspirin and Persantin (dipyridamole) are well-tolerated antiplatelet agents. Although aspirin is a relatively weak platelet inhibitor, its efficacy and safety is overwhelming and its cost-effectiveness in the management of cardiovascular disease unparalleled. Aspirin in its usual low-dose formulation irreversibly blocks platelet cyclo-oxygenase, but not extraplatelet prostaglandin production by, for example, macrophages and proliferating smooth muscle cells. Persantin is an antiplatelet agent with a questionable efficacy. Although it is probably safe, its main use is as an intravenous drug to precipitate myocardial ischaemia for diagnostic purposes.

Abciximab

James J Ferguson and Paul Kim

Introduction

The purpose of this chapter is to summarize the currently available clinical data on abciximab (c7E3 Fab, ReoPro), a chimeric monoclonal antibody directed at platelet glycoprotein (GP) IIb/IIIa. Since its commercial release in January 1995, there has been considerable controversy about the appropriate clinical role for this potent form of antiplatelet therapy. This chapter provides background information on the development of abciximab, data from recent randomized clinical trials presents some areas of current clinical controversy, and explores some of the potential future applications of abciximab.

Background/development

Platelet glycoprotein IIb/IIIa antagonists are a novel class of therapeutic compounds that inhibit platelets by blocking the final common pathway of platelet aggregation. A variety of antibody, peptide, and nonpeptide compounds have been developed. Several of these agents have been studied in recent clinical trials; the most extensively investigated and the first to enter the commercial marketplace in the USA is abciximab, a chimeric human murine antibody Fab fragment.

Coller was the first to describe the use of a murine monoclonal antibody (m7E3) directed

against glycoprotein IIb/IIIa.^{1,2} Subsequent studies³⁻¹⁰ utilized the Fab fragment of this antibody, in the hope that removing the Fc region would reduce the potential for complement activation and decrease the accompanying risk of thrombocytopenia resulting from clearance of antibody-coated platelets by the reticuloendothelial system. However, both the murine antibody and the murine Fab fragment were associated with a high incidence of development of human antimurine antibodies. To reduce the potential immunogenicity of this form of IIb/IIIa-targeted therapy, the DNA sequences for the constant domains of the murine antibody were replaced with corresponding human DNA sequences, leaving intact variable-region murine sequences.¹¹ The resulting chimeric compound, abciximab, contains approximately 50% human sequences (Figure 8.1).

The Investigational New Drug application for abciximab was filed in February 1990. Phase I dose-escalation trials in normal volunteers were conducted later that year. This was followed in February 1991 by Phase II trials in high-risk patients undergoing percutaneous transluminal coronary angioplasty (PTCA). The pivotal Phase III study, EPIC, was initiated in November 1991, and the results were first presented at the American College of Cardiology Scientific Sessions in March 1993. The commercial product (ReoPro, Centocor and

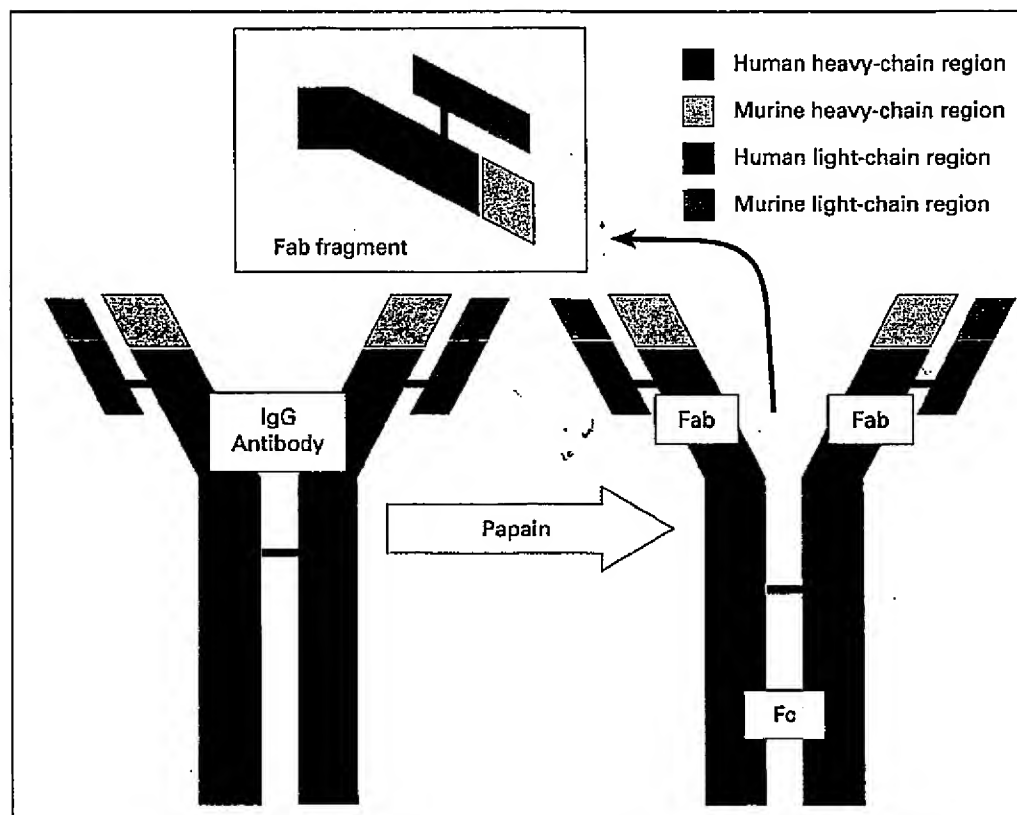


Figure 8.1

The derivation of the Fab fragment of a chimeric human-murine antibody directed at platelet GPIIb/IIIa

Eli Lilly & Co.) was launched after FDA approval in early 1995.¹² Subsequent large clinical trials (described later) included EPILOG¹³, CAPTURE¹⁴, RAPPORT¹⁵, ERASER¹⁶, and EPISTENT¹⁷. Commercial acceptance of the product was initially guarded, with enthusiastic use at some centers and more gradual incorporation of the new agent into clinical practice in others. With time, however, the clinical uptake and use of abciximab has con-

tinued to grow. At the beginning of 1995, ReoPro was used in approximately 45% of coronary interventions performed in the US.

Pharmacokinetics and pharmacodynamics

The pharmacokinetics of abciximab have been described in some detail.¹² Free drug is clear-

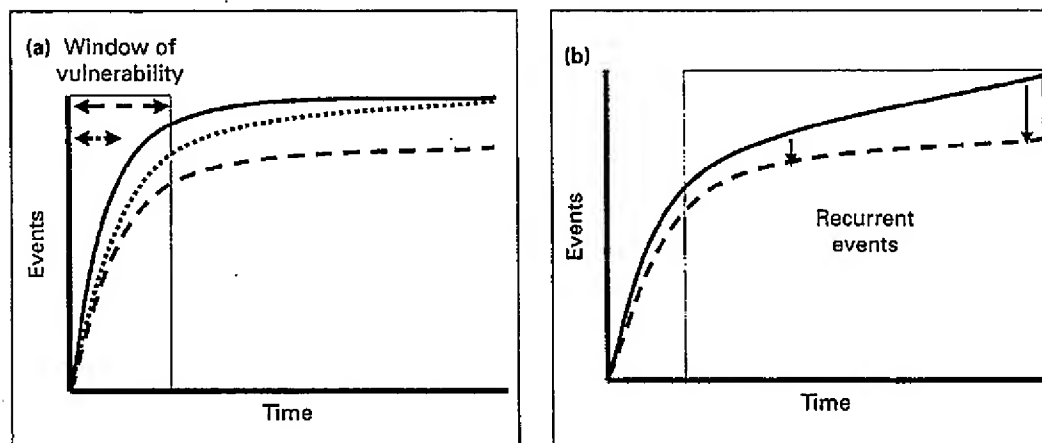


Figure 8.11

(a) Initial benefit during the 'window of vulnerability'. If the treatment duration adequately covers the vulnerable period, the benefits will be sustained. If it does not, there may be loss of benefit over the subsequent time period. (b) Recurrent events, outside the 'window of vulnerability' depend on long-term therapeutic mechanisms, such as reducing the likelihood of plaque rupture, and/or reducing the likelihood of thrombus formation.

dynamic effects, however, it seems ideally suited to covering as much as possible of the 'window of vulnerability' associated with injury to the vessel wall.

Summary

In this chapter we have summarized the data currently available on the use of abciximab, both in the catheterization laboratory and in

other clinical scenarios. The use of this potent platelet inhibitor has evolved substantially over the last 3–4 years, and several very exciting applications are looming on the horizon. The real challenge for the future will come as we begin to interdigitate abciximab into existing treatment algorithms, and combine it with low-molecular-weight heparin, new thienopyridines plus aspirin and, possibly, with oral forms of the GPIIb/IIIa antagonists.

Peptide and non-peptide inhibitors of platelet glycoprotein IIb/IIIa

Robert A Harrington and John Alexander

Atherosclerosis, vascular injury, and coronary thrombosis

Formation and progression of atherosclerotic plaque involves a complex interaction of lipids, inflammation, thrombosis, and mechanical shear forces.¹ The pathologic underpinnings of acute coronary syndromes (ACS), including unstable angina and acute myocardial infarction, and the acute complications of percutaneous coronary intervention both result from the rupture, fissuring, or erosion of

an atherosclerotic plaque, with subsequent intracoronary thrombosis formation (Figure 9.1).² In the setting of an ACS, plaque injury is spontaneous and unpredictable; however, in coronary intervention, vascular injury is induced by the coronary interventionist in an attempt to expand the obstructed coronary lumen. Following either spontaneous or planned plaque injury, acute thrombosis of a coronary vessel leads to subtotal or total vascular occlusion with interruption of normal blood flow, causing myocardial ischemia and, if persistent, necrosis.

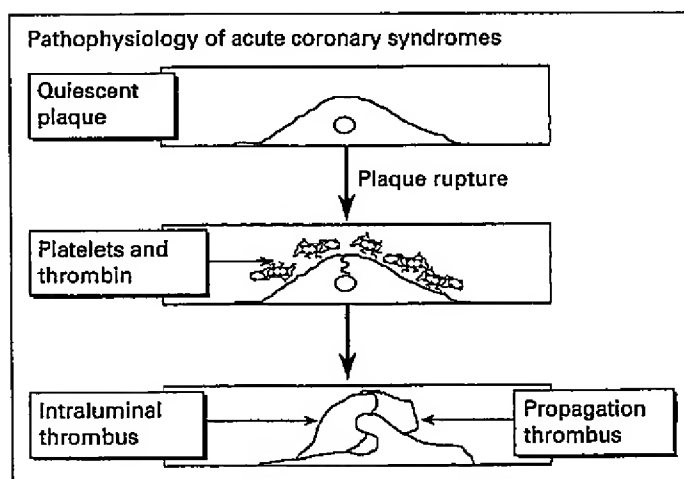


Figure 9.1
Pathophysiology of acute coronary syndromes.

The initial hemostatic response to such vascular injury is platelet deposition over the injured endothelium. Activation of platelets by any of a number of agonists, including adenosine diphosphate (ADP), epinephrine (adrenaline), collagen, thrombin, or shear stress, leads to ongoing platelet activation and aggregation. The resulting core of platelet-rich thrombus provides the necessary phospholipid surface on which assembly of the prothrombinase complex occurs, precipitating the conversion of prothrombin to thrombin, which then catalyzes the conversion of fibrinogen to fibrin.³ In addition to its role in the formation of fibrin, thrombin, as the central enzyme of the coagulation cascade, activates factors V, VIII, and XIII, activates platelets, and controls its own regulation through its effects on thrombomodulin and protein C.⁴

Rationale for antithrombotic therapies in the acute ischemic coronary syndromes

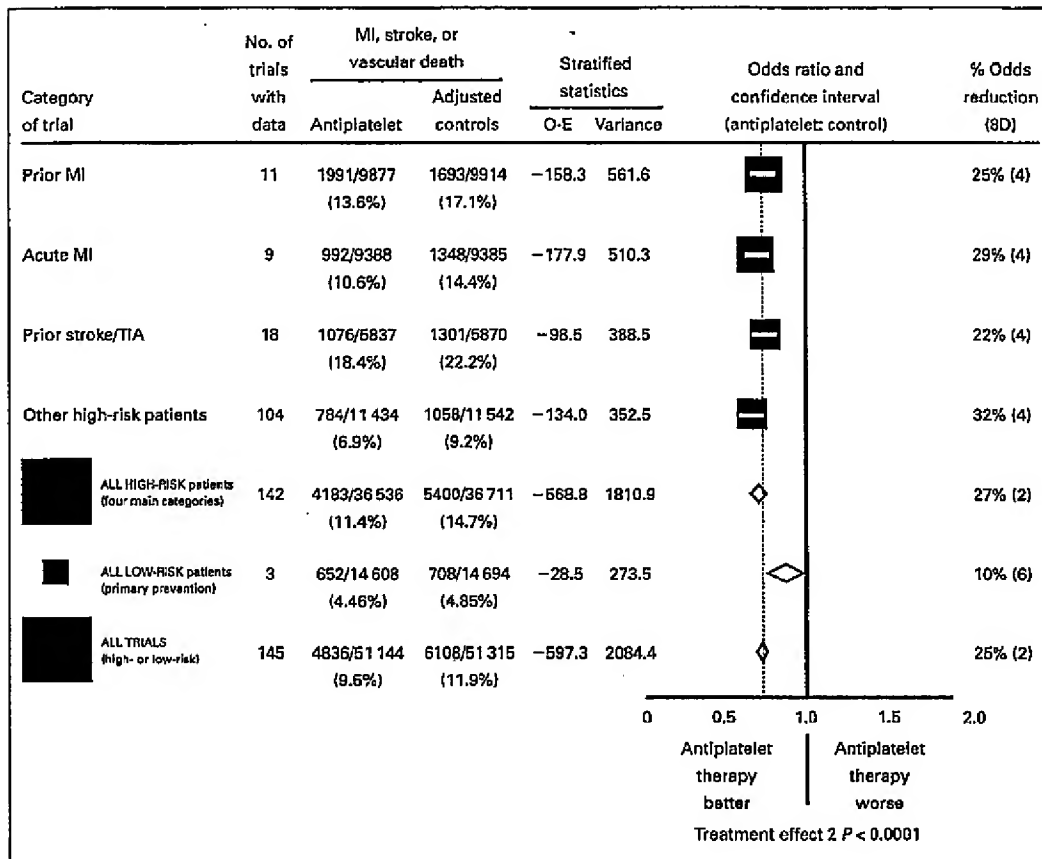
Given the central role of thrombosis in the pathophysiologic mechanism of unstable angina and acute myocardial infarction, the use of antithrombotic agents in the treatment of patients who present with acute ischemia seems reasonable. Oral antiplatelet therapy with aspirin provides impressive clinical benefits to patients presenting with a spectrum of vascular diseases, and reduces the risk of death, myocardial infarction, and stroke by approximately one-third (Figure 9.2).^{5,6} Recently, the antiplatelet agent clopidogrel, which inhibits ADP-induced activation of platelets, was shown to be marginally superior to aspirin alone in reducing the risk from the composite of vascular death, myocardial infarction, and stroke in patients with an array of atherosclerotic vascular disease.⁷

Despite the wide use of antiplatelet therapy, primarily with aspirin, in patients with atherosclerotic disease, there remains significant morbidity and mortality associated with acute ischemia; thus, there is a clinical need for alternative antiplatelet therapies. Aspirin, ticlopidine, and clopidogrel are all relatively weak antiplatelet agents. Additionally, there is a group of patients, perhaps as high as 15–30% of patients treated with aspirin, who are non-responders or 'resistant' to the antiplatelet effects of aspirin and who appear to be at increased risk for subsequent vascular events.^{8–10}

For these reasons, recent research has focused on the development of new, more potent antiplatelet therapies as a means of combating the problem. Platelet aggregation occurs via fibrinogen binding to the cellular receptor, glycoprotein (GP) IIb/IIIa, on adjacent platelets. This receptor belongs to a family of integrins that are involved in the processes of cellular adhesion. On circulating and quiescent platelets, the GPIIb/IIIa receptor is incapable of binding other circulating molecules like fibrinogen or von Willebrand factor (i.e. it is ligand-unresponsive). After activation, however, the platelet GPIIb/IIIa receptor undergoes a conformational change that permits the binding of adhesion molecules, primarily fibrinogen, and facilitates platelet aggregation. Interfering with the ability of platelet GPIIb/IIIa receptors to bind fibrinogen thus inhibits the 'final common pathway' of platelet aggregation and provides an attractive target for a cardiovascular therapeutic agent.

Platelet GPIIb/IIIa inhibition

Coller et al¹¹ reported on the first experience with a murine monoclonal antibody that

**Figure 9.2**

Proportional effects of antiplatelet therapy (145 trials) on vascular events (myocardial infarction, stroke, or vascular death) in four main high-risk categories of trial and in low-risk categories (primary prevention). (Stratified ratio of odds of an event in treatment groups to that in control groups is plotted for each group of trials (black square) along with its 99% confidence interval (horizontal line). Overviews of results for certain subtotals (and 95% confidence intervals) are represented by diamonds. Odds reductions observed in particular groups of trials are given to right of solid vertical line.) TIA, transient ischemic attack; MI, myocardial infarction.

almost completely inhibited the ability of platelets to aggregate. Refinement of this initial work led to the development of the chimeric monoclonal antibody fragment,

abciximab.¹² In a series of trials, studying a variety of patient populations and interventional strategies, abciximab was then shown to be effective in reducing the acute ischemic

complications in a broad spectrum of patients undergoing percutaneous coronary intervention.¹³⁻¹⁷ Abciximab is discussed extensively in Chapter 8.

In addition to abciximab, several other intravenous GPIIb/IIIa inhibitors have been developed. These are commonly referred to as 'small-molecule inhibitors', to contrast them with the monoclonal antibody GPIIb/IIIa inhibitor, abciximab. In extensive clinical trial work, including over 33 000 randomized patients, the intravenous GPIIb/IIIa inhibitors have been demonstrated to reduce acute ischemic complications in patients undergoing percutaneous coronary intervention and in patients presenting with non-ST-segment elevation ACS.¹⁸ The remainder of this chapter will focus on the intravenous small molecule inhibitors of the GPIIb/IIIa receptor. Specific clinical indications are discussed separately in Chapters 12-17.

Small-molecule GPIIb/IIIa inhibitors

Around the same time that Collier¹¹ was working on a monoclonal antibody approach to GPIIb/IIIa antagonism, other investigators were attempting to interfere with the GPIIb/IIIa receptor through small molecule inhibitors. These agents, both peptides and nonpeptide mimetics, are small-molecule inhibitors that were developed using the knowledge that fibrinogen binding to the receptor occurs via RGD amino acid sequences (arginine-glycine-aspartic acid). Key differences between the small-molecule inhibitors and the monoclonal antibody GPIIb/IIIa antagonist can be seen in Table 9.1. Three intravenous small-molecule inhibitors are currently under clinical development, eptifibatide, tirofiban, and lamifiban. Eptifibatide and tirofiban have been approved for clinical

Properties	Monoclonal antibody fragment	Small-molecule inhibitors
Chemical nature	Antibody	Peptides and peptidomimetics
Size	Large (approximately 48 kD)	Small (<1 kD)
Onset of platelet aggregation inhibition	Rapid	Rapid
Plasma half-life	Very short (<10 min)	Moderate (1-2 h)
Reversibility of platelet aggregation inhibition	Slow	Rapid
Binds non-GPIIb/IIIa integrins	Yes	No (GPIIb/IIIa specific)
Antigenicity	Yes (human anti-chimeric antibodies)	No

GP, glycoprotein.

Table 9.1
Similarities and differences among clinically available GPIIb/IIIa.

<i>GPIIb/IIIa Antagonists</i>	<i>Approved indications</i>	<i>Major clinical trials</i>
Eptifibatide (Integrilin TM)	PCI Non ST-segment elevation ACS	IMPACT II ²⁰ (<i>n</i> = 4010): Elective and high-risk PCI PURSUIT ^{21,25} (<i>n</i> = 10,948): Non ST- segment elevation ACS
Tirofiban (Aggrastat TM)	Non ST-segment elevation ACS	RESTORE ²² (<i>n</i> = 2141): High-risk PCI PRISM ^{23,25} (<i>n</i> = 3232): Non ST- segment elevation ACS PRISM-PLUS ^{24,25} (<i>n</i> = 1915): Non ST-segment elevation ACS
Lamifiban	Phase III trials	PARAGON A ²⁶ (<i>n</i> = 2282): Non ST-segment elevation ACS PARAGON B (<i>n</i> = 4000 planned): Non ST-segment elevation ACS (ongoing trial)

PCI, percutaneous coronary intervention; ACS, acute coronary syndromes

Table 9.2

Small-molecule GPIIb/IIIa inhibitors: approved indications and supporting clinical trials.

use in the USA, while lamifiban is in Phase III clinical trials. Table 9.2 lists the agents and summarizes the approved indications and randomized trials of more than 1000 patients.²⁰⁻²⁵

Abciximab binds non-specifically to the GPIIb/IIIa receptor in an irreversible manner and consequently, even after the infusion is terminated, platelet function is maximally (>80% inhibition of platelet aggregation) inhibited for many hours and measurably altered for days to weeks. In addition, because of its non-specificity, abciximab may have other effects on platelet function, the importance of which is not currently understood. In contrast, the small-molecule inhibitors are highly specific competitive antagonists of the GPIIb/IIIa receptor. Thus, after terminating an infusion of one of the short-acting small-mole-

cule inhibitors, platelet function (as measured by inhibition of platelet aggregation) rapidly returns to normal as the competitive antagonist is cleared from the circulation. It is unknown whether such differentiating characteristics as the non-specificity and prolonged duration of the antiplatelet effect translate into differing degrees of clinical benefit. In a recent systematic overview of all randomized clinical trials, including more than 1000 patients and performed with the four major intravenous GPIIb/IIIa antagonists, regardless of agent, the point estimate for clinical benefit consistently favors the platelet inhibitor to a similar degree.¹⁸ In the absence of direct head-to-head trials comparing abciximab and the small-molecule inhibitors, speculation about differential clinical effects is premature.

Small-molecule antagonists: clinical development

Percutaneous coronary intervention and the ACS were chosen as the rational areas in which to develop eptifibatide, tirofiban, and lamifiban, given the pivotal role that the platelet plays in the pathophysiology of plaque rupture (whether induced or spontaneous) and subsequent arterial thrombosis. Perceived advantages to the small-molecule inhibitors, compared with the monoclonal antibody inhibitor, included their rapid reversibility, their specificity for GPIIb/IIIa and not other cellular integrins, and their likely lack of immunogenicity. Rapid reversibility appeared to be an especially attractive safety feature in an era of balloon angioplasty, without coronary stenting, when more than 5% of patients would require emergency bypass surgery to salvage a failed percutaneous procedure. In addition, rapid recovery of platelet function was felt to be necessary if these agents were to be used as empirical therapy for patients presenting with ACS. At the time of patient presentation, the clinician must make a treatment decision without knowing the patient's coronary anatomy and, consequently, before knowing whether the patient would require surgical intervention.

Eptifibatide

Eptifibatide (Integrilin™, COR Therapeutics, South San Francisco) is the prototypical peptide inhibitor of platelet GPIIb/IIIa. It is a cyclic heptapeptide modeled on the structure of barbourin, a specific inhibitor of GPIIb/IIIa found in the venom of the Southeastern pygmy rattlesnake. Replacing lysine for arginine results in a KGD sequence that provides eptifibatide with high affinity and specificity for the GPIIb/IIIa receptor.²⁶ Eptifibatide is cleared

predominantly through renal mechanisms and has a circulating plasma half-life of approximately 150 min. Inhibition of platelet aggregation, in response to a variety of agonists, is dose-dependent and predictable based on plasma concentrations.^{27,28} Preclinical experience with animal models of arterial thrombus showed eptifibatide to be a potent agent at inhibiting platelet-dependent arterial thrombosis.²⁹ Studies in normal human volunteers demonstrated that eptifibatide had a rapid onset of action and was readily reversible with a calculated plasma half-life in normals of approximately 1 h.²⁹

Eptifibatide development: percutaneous coronary intervention

Although the first Phase II trial with eptifibatide was in patients with unstable angina,³⁰ percutaneous coronary intervention was the area chosen for the first full development trial with this new agent. The acute ischemic complications of these procedures include death, myocardial infarction, and the need for repeat urgent procedures. These complications were believed to be, at least in part, platelet-mediated events, as demonstrated by the benefits conferred by aspirin in lowering the risk of these complications.⁵ This chapter will review the highlights of the completed trials with all three small molecule inhibitors of GPIIb/IIIa. Details of the benefits of GPIIb/IIIa inhibition in patients undergoing percutaneous coronary intervention can be found in Chapter 7.

Two small, Phase II, dose-finding trials were completed with eptifibatide before a large Phase III project in coronary intervention: IMPACT (Integrilin to Manage Platelet Aggregation and Prevent Coronary Thrombosis)²⁷ and IMPACT Hi-Lo.²⁸ The IMPACT trial enrolled 150 patients undergoing elective

to the discretion of the investigator. In an open-label fashion, a dose of lamifiban was identified that provided greater than 85% ADP-induced inhibition of platelet aggregation; patients were then randomized to receive this dose of lamifiban or placebo, along with the fibrinolytic agent. Those receiving t-PA also received heparin, while those treated with streptokinase did not.

In the PARADIGM study, the highest dose of lamifiban (400 µg bolus + 2.0 µg-min infusion for 48 h) resulted in a 91% median (interquartile range, 84–95%) inhibition of aggregation at steady-state measurement. There was also an improvement in myocardial reperfusion as measured by continuous ST-segment monitoring with less time to ST-segment resolution and less time to ST-segment steady state. Both these variables were shown in previous trials to correlate with an improvement in 30-day clinical outcomes.⁴⁹ There was an increased risk of bleeding among patients treated with lamifiban compared with placebo, although there were too few patients to make reliable observations about the risk of intracranial hemorrhage associated with the combination strategy.

Unresolved issues

A systematic overview of the trials with the small molecule GPIIb/IIIa antagonists supports the hypothesis that this class of agent benefits patients presenting with ACS without persistent ST-segment elevation (Figure 9.3).¹⁸ The benefits of the small-molecule inhibitors also appear to extend to coronary intervention, although only eptifibatide is approved for use in this indication. More work is required on the potential role of these agents in patients with ACS with persistent ST-segment elevation.

While these agents clearly represent a major therapeutic advance for the treatment of patients with acute ischemic heart disease, many questions remain regarding their use. Firstly, there are similarities and differences among the three small-molecule inhibitors. Whether these differences are clinically important is unknown; this question will only be answered using head-to-head comparative trials. Without such trials, speculation about meaningful differences is inappropriate, given the major differences in clinical trial design even within a common indication. Similarly, the question of whether the small-molecule inhibitors and the monoclonal antibody antagonist are clinically different is unknown; determining the answer likewise requires head-to-head trials.

Secondly, the optimal dosing strategy for each of these agents remains unknown. Future trials will need to consider using point-of-care platelet aggregation testing⁵⁰ to define better the level of inhibition achieved in individual patients. Studies also need to be performed to determine more accurately the level of platelet inhibition that is associated with maximum benefit.⁴⁶ PARAGON B, an ongoing study with lamifiban in patients with ACS without persistent ST-segment elevation is testing whether a dosing strategy that takes into consideration individual patient characteristics such as age, gender, weight, and renal function, can lead to more optimal drug concentrations and to improved patient outcomes.

Finally, in addition to these GPIIb/IIIa inhibitors, there exist fibrinolytics and novel antithrombins, including low-molecular-weight heparins and direct thrombin inhibitors that are approved for use or being studied for use in patients with acute ischemic heart disease. The combination of potent antiplatelet therapies with novel antithrombins and fibrinolytics needs further investigation. The sma

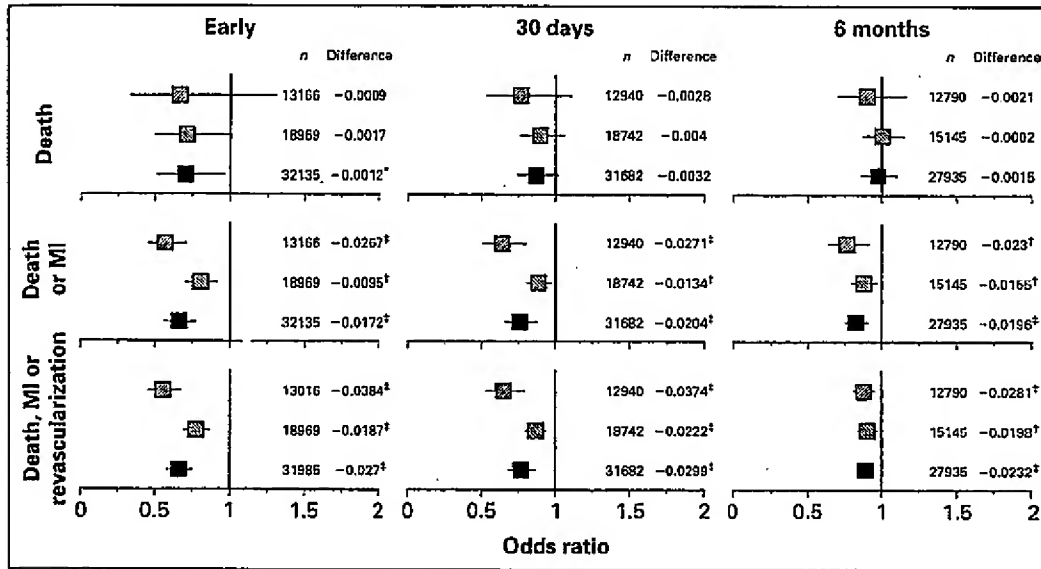


Figure 9.3

Odds ratios (OR) and 95% confidence intervals (CI) for risk of death, death or myocardial infarction (MI), and death, MI, or revascularization (Revasc) 48 to 96 h, 30 days, and 6 months after randomization to a glycoprotein IIb/IIIa inhibitor (versus placebo). ORs are given for combined percutaneous intervention trials (▨), combined non-ST-segment elevation acute coronary syndromes trials (▩), and all collected trials (■). n, sample size; Difference, risk difference. * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$.

dose-finding studies using GPIIb/IIIa antagonists and full-dose fibrinolytic therapy appear promising. Several trials are underway with lower doses of the fibrinolytic agents that, in combination with GPIIb/IIIa inhibitors,

improve angiographically determined myocardial perfusion. Much larger trials, enrolling tens of thousands of patients, are needed to show the safety and efficacy of this approach.

Oral platelet glycoprotein IIb/IIIa blockers

Christopher P Cannon

Introduction

Every year more than four million patients are admitted to hospitals worldwide with the diagnosis of unstable angina or acute myocardial infarction (MI).^{1,2} The initiating event of unstable coronary syndromes is atherosclerotic plaque rupture followed by local thrombosis.³ Antithrombotic therapy, currently aspirin and heparin, is the mainstay of therapy, and numerous trials have documented a dramatic 25–50% benefit on death and/or MI with aspirin in acute coronary syndromes.⁴

The importance of *antiplatelet* therapy comes from the broad experience with aspirin, which has dramatic effects in reducing both mortality and non-fatal events in patients across the spectrum of acute coronary syndromes.^{4–11} In addition, the newer class of antiplatelet agents, the thienopyridines (ticlopidine and clopidogrel), which inhibit platelet function are stronger antiplatelet agents than aspirin, have been shown to be beneficial in reducing clinical events compared with aspirin alone in coronary stenting,^{12–14} and in symptomatic patients with atherosclerosis.^{4,15–17} This wealth of data has focused attention on the platelet, as a target for more potent therapies, notably the inhibitors of the platelet glycoprotein (GP) IIb/IIIa, which mediates platelet aggregation.

Glycoprotein IIb/IIIa blockers

GPIIb/IIIa blockers are a new, potent class of platelet inhibitors. GPIIb/IIIa receptor antagonists block the binding of fibrinogen to specific membrane GPIIb/IIIa integrin receptors, thus preventing platelet aggregation induced by various platelet agonists.^{18,19} Platelet GPIIb/IIIa is a member of the integrin receptor superfamily of complexes that mediate cell–protein and cell–cell interactions.¹⁸ GPIIb/IIIa is a calcium-dependent heterodimer, composed of two different subunits (α_{IIb} and β_3), both of which span the platelet membrane. The GPIIIa subunit contains a four-amino acid sequence, which is crucial for binding of fibrinogen and other ligands.¹⁸ The first three amino acids are arginine-glycine-aspartic acid (RGD) while the fourth amino acid may vary. Low-molecular-weight peptide and non-peptide GPIIb/IIIa inhibitors have been developed to bind to the RGD sequence of the receptor, thereby interfering with the binding of fibrinogen to GPIIb/IIIa.

Mechanisms of action of ASA, ticlopidine, and GPIIb/IIIa inhibitors

The mechanisms of action of the current antiplatelet agents and GPIIb/IIIa inhibitors are quite distinct. Aspirin permanently acetylates cyclo-oxygenase, thereby blocking the

synthesis of thromboxane A_2 (TXA₂) by the platelet.²⁰ By decreasing the amount of TXA₂ released, which would act to stimulate other platelets, there is a decrease in overall platelet aggregation. This inhibition of cyclooxygenase is permanent, thus the antiplatelet effects last for the lifetime of the platelets, that is, between 7 and 10 days.

The thienopyridine class of agents (ticlopidine and clopidogrel) are believed to act by blocking the ADP receptor.^{17,21,22} They may also inhibit intracellular processing of activation of the ADP pathway but have no effect on the numerous other stimulants to platelet aggregation, such as thrombin and collagen.²² Their onset of antiplatelet effect is delayed, with peak inhibition occurring 2–4 days after the start of therapy, although clopidogrel has more rapid onset.

In contrast, GPIIb/IIIa inhibitors bind to the GPIIb/IIIa receptor and thereby block the final common pathway of platelet aggregation. By binding to the receptor, they prevent the binding of fibrinogen to the platelet and thereby prevent formation (or progression) of a platelet plug. Thus, no matter what stimuli to platelet activation exist, the platelet is inhibited by the GPIIb/IIIa inhibitor—making it a great deal more able to inhibit platelet aggregation than aspirin (or ticlopidine). When testing platelet aggregation in the laboratory, aspirin inhibits ADP-induced platelet aggregation by approximately 10%, ticlopidine and clopidogrel by approximately 30–40%,²³ and the doses of the GPIIb/IIIa inhibitors being tested clinically inhibit platelet aggregation by approximately 80–90%.²⁴ Several potential mechanisms exist to explain how GPIIb/IIIa inhibition may improve clot resolution and clinical outcome in patients with acute coronary syndromes. Firstly, by blocking platelet aggregation in the platelet-rich arterial thrombus, propagation of the thrombus is prevented;

GPIIb/IIIa inhibitors may also *disaggregate* recently-formed platelet plug. Second, by preventing accumulation of a large number of platelets at the lesion, it decreases the amount of platelet phospholipid membrane, a cofactor for thrombin generation and of the clotting cascade. Thirdly, a thrombus rich in platelets may resist thrombolysis (either thrombolytic therapy or endogenous thrombolysis) owing in part to the increased presence of plasminogen activator inhibitor (PAI-1), a potent natural inhibitor of fibrinolysis that exists in high concentrations in platelets.

Potential risks of GPIIb/IIIa inhibition

Bleeding

The major concerns with any antithrombotic agents are bleeding, and for platelet inhibitors, thrombocytopenia. As with any antithrombotic agent, the potential for increased bleeding exists. Although the initial EPIC study showed increased bleeding using abciximab plus heparin during angioplasty compared with heparin alone,²⁵ a strong interaction with the dose of heparin was observed, such that in the EPILOG trial, the rate of major bleeding was identical in heparin control patients and in those receiving abciximab and low-dose heparin.²⁶ Similarly, the rate of major bleeding has generally not been found to increase significantly in other trials using intravenous^{27,28} or oral GPIIb/IIIa inhibitors.²⁹ Thus, the use of lower doses of heparin and careful monitoring of the level of anticoagulation will avoid bleeding complications in patients receiving GPIIb/IIIa inhibitors. With regard to monitoring the degree of platelet inhibition, trials to date have used fixed dosing; however, investigation is currently underway to determine

when and where monitoring of platelet function may be clinically useful.³⁰

Thrombocytopenia

Thrombocytopenia is the other important side-effect of GPIIb/IIIa inhibition. Platelet counts falling below 100 000 occur in approximately 1–2% of patients treated with GPIIb/IIIa inhibitors, while platelet counts falling to below 50 000 occurs in less than 0.5% of patients.^{26,28,31} In the initial trials, thrombocytopenia generally occurred on either the first day after beginning therapy, or after approximately 2 weeks' of therapy. The mechanism by which it occurs is not defined completely, but appears to involve immune mechanisms. Fortunately, it is nearly always reversible, with platelet counts returning to normal after a few days.

Types of GPIIb/IIIa inhibitors

There are three broad categories of GPIIb/IIIa inhibitors:

- (1) The Fab fragment of a monoclonal antibody to the GPIIb/IIIa receptor, abciximab (ReoPro[®]) (see Chapter 8);
- (2) The intravenous peptide and non-peptide small-molecule inhibitors, such as eptifibatide (Integrilin[®]) and tirofiban (Aggrastat[®]) (see Chapter 4);
- (3) The oral GPIIb/IIIa inhibitors, such as xemilofiban, orbofiban, sibrafiban, roxifiban and many others.

Abciximab

Abciximab, the monoclonal antibody, binds very tightly to the GPIIb/IIIa receptor.³² Thus, the antiplatelet effect lasts much longer than the infusion period—a potential benefit on improving efficacy. Conversely, if bleeding occurred, stopping the drug will not reverse

the antiplatelet effect immediately; transfusion of platelets however, will allow the antibodies to redistribute among all the platelets, thereby reducing the level of platelet inhibition. Abciximab also binds to other integrins on the platelet receptor, such as the vitronectin receptor¹⁸ but the clinical significance of this cross-reactivity is not yet established.

Peptide and non-peptide small-molecule inhibitors

The peptide and peptidomimetic inhibitors (e.g. tirofiban and eptifibatide) are competitive inhibitors of the GPIIb/IIIa receptor.^{33,34} Thus, the level of platelet inhibition is directly related to the drug level in the blood. Since both inhibitors have short half-lives, when the drug infusion is stopped,^{33,34} the antiplatelet activity reverses after a few hours, which is potentially beneficial to avoid bleeding complications. Conversely, for prolonged antiplatelet effect, the drug needs to be given intravenously for a longer period of time. The inhibitors developed to date have been targeted specifically to the GPIIb/IIIa receptor and not to cross-react with other integrins.

Oral GPIIb/IIIa inhibitors

The third group of GPIIb/IIIa inhibitors are the oral agents. These agents are also competitive inhibitors, and are usually pro-drugs, which are absorbed and then converted to active compounds in the blood.^{29,35,36} The oral agents all have longer half-lives than the intravenous compounds and, as such, they can be given once, twice or three times daily (depending on the half-life) in order to achieve relatively steady levels of GPIIb/IIIa inhibition. With oral dosing, long-term therapy (i.e. over 1 year) is possible. However, the long half-life also means that, if bleeding occurs, the drug must be removed from the circulation in order to reduce the antiplatelet effect. Currently this

can be accomplished acutely using hemodialysis or charcoal hemoperfusion. The development of specific antidotes would be an attractive alternative method for removing the drug quickly from the circulation.

Initial clinical trials with intravenous GPIIb/IIIa inhibitors

Numerous trials have shown that intravenous GPIIb/IIIa inhibitors are beneficial in acute settings (see Chapters 8 and 9).^{25-28,31,37-40} In studies of intravenous GPIIb/IIIa antagonists in coronary angioplasty and unstable angina, there have been significant reductions in recurrent ischemic events. For example, in the PRISM-PLUS trial, patients with unstable angina or non-Q-wave MI treated with aspirin, heparin, and a 2-4-day infusion of tirofiban had a 32% reduction in death, MI, or refractory ischemia at 7 days compared with placebo (12.9% versus 17.9%, $p = 0.004$), and a 30% risk reduction in death or MI at 30 days ($p = 0.03$).³¹ A significant improvement in death or MI was also observed in the larger PURSUIT trial with a 3-day infusion of eptifibatide.³⁸ A logical extension of the potent form of therapy is to develop strategies for more sustained or chronic GPIIb/IIIa inhibitor with long-term treatment. Thus, the hypothesis for current trials is that oral GPIIb/IIIa inhibitors will provide a novel form of therapy to prevent early and late recurrent thrombotic complications in patients with acute coronary syndromes or those undergoing percutaneous coronary intervention (PCI).

Rationale for long-term GPIIb/IIIa inhibition

The rationale for long-term platelet inhibition comes from both biological and clinical observations. From the biological standpoint, as already discussed, platelet function tests show that platelets remain activated long after patient is stabilized clinically. Active thrombus has been observed by coronary angiography even 1 month following acute coronary syndromes,⁴¹ indicating the long period of time that is needed for complete antithrombotic treatment of a culprit lesion. Similarly, in the TIMI 12 trial of an oral GPIIb/IIIa inhibitor in patients stabilized after an acute coronary syndrome, we observed high levels of activated platelets in patients at the start of the study but also 1 month later (Table 10.1) despite oral GPIIb/IIIa treatment.⁴² Thus, there is an active, prothrombotic 'milieu' in patients following acute coronary syndromes, which could potentially benefit from more aggressive antithrombotic therapy than just aspirin.

From the clinical standpoint, one observation supporting a potential role for long-term GPIIb/IIIa inhibition is that greater benefit appears to be achieved with longer duration of GPIIb/IIIa inhibition. When contrasting the statistically significant results obtained with abciximab use during angioplasty^{25,26,39,40} with the loss of early benefit in angioplasty trials seen after the shorter infusions (24-36 h) of tirofiban and eptifibatide,^{27,28} abciximab had very long duration of action on the platelet with antiplatelet activity detected up to 1-weeks after administration.⁴⁴ This suggests that the prolonged antiplatelet effect of abciximab may be responsible for some of its sustained beneficial effect.

Further support for this hypothesis comes from the RESTORE and PRISM-PLUS trials. In the RESTORE trial, tirofiban was admini-

Agonist	Baseline (%)	Day 7 (%)	Day 28 (%)
Spont	28 ± 19	17 ± 13	20 ± 16
Oral ADP	36 ± 17	24 ± 17	26 ± 18
Oral ADP	48 ± 19	24 ± 20	26 ± 17
Oral ADP	65 ± 19	168 ± 18	64 ± 18

$P < 0.001$ vs. $P < 0.05$ vs. $P < 0.001$
 Data from ADP challenge

Table 10.1

P Selectin results from the TIMI 12 study.

tered for 36 h, and a 26% reduction in MI and no difference in death at 30 days was observed. In the PRISM-PLUS trial, in which patients received 48–72 h therapy with tirofiban, those undergoing PTCA had a 45% reduction in death or MI at 30 days.^{27,31} These comparisons are not randomized but are consistent with the hypothesis that a longer duration of GPIIb/IIIa inhibition is more beneficial than a shorter duration of platelet inhibition.

The second clinical observation is that the benefit of intravenous GPIIb/IIIa inhibitors is achieved *only during the infusion*. Owing to the potent platelet inhibition, the benefits are maintained but no *added* benefit is observed after the infusions are stopped. For example, in the PURSUIT trial in unstable angina and non-Q-wave MI, eptifibatide reduced death or MI by an absolute 1.7% at 72 h; the reduction was similar (1.5%) at 30 days.³⁸ Similarly, in PRISM-PLUS, tirofiban plus heparin reduced death or MI by 3.4% at 7 days, and by 3.2% at 30 days.³¹ It is hypothesized that chronic dosing with oral GPIIb/IIIa antagonists will demonstrate ongoing benefit throughout the period of treatment and thereby amplify the

benefits seen to date with the intravenous GPIIb/IIIa inhibitors.

Potential role for long-term oral GPIIb/IIIa inhibition

Oral GPIIb/IIIa receptor antagonists offer the potential for long-term treatment with many possible clinical applications (Table 10.2). Potential applications include:

- (1) The early phase of acute coronary syndromes;
- (2) Secondary prevention of events after stabilization from an acute coronary syndrome; and
- (3) Both acute treatment and secondary prevention.

By extension, oral GPIIb/IIIa inhibitors could also potentially inhibit the development of athero(thrombo)sclerosis, which is sometimes augmented by microthrombotic events within the plaque. In addition, these agents may be useful for percutaneous coronary intervention, and also in stroke, for both early treatment and secondary prevention.

Treatment	Agent
<ul style="list-style-type: none"> • Acute treatment <ul style="list-style-type: none"> • Percutaneous coronary intervention • Unstable angina/non-ST elevation MI <ul style="list-style-type: none"> ■ ? ST-elevation MI with thrombolysis ■ ? Stroke—? ■ ? Peripheral vascular disease ■ ? Cerebrovascular intervention 	Intravenous GPIIb/IIIa inhibitors Oral GPIIb/IIIa inhibitors —? Intravenous + oral inhibitors
<ul style="list-style-type: none"> • Secondary prevention <ul style="list-style-type: none"> ■ ? Percutaneous coronary intervention ■ ? Post-acute coronary syndromes ■ ? Stroke ■ ? Peripheral vascular disease 	? Oral GPIIb/IIIa inhibitors
<ul style="list-style-type: none"> • Both early treatment and secondary prevention • Inhibition of athero(thrombo)sclerosis 	
* Current indications for GPIIb/IIIa inhibition are listed in bold type.	

Table 10.2

Current and future indications for GPIIb/IIIa inhibition.*

Initial clinical experience

Pharmacokinetics and pharmacodynamics

Several orally active platelet GPIIb/IIIa inhibitors have been studied in clinical trials (Table 10.3). These agents are usually in a pro-drug form and require hepatic conversion to an active moiety. Absolute bioavailability is generally low as shown in Table 10.3. Currently available data suggest these agents produce inhibition of *ex vivo* platelet aggregation in response to various agonists (e.g. ADP, collagen, thrombin receptor activating peptide (TRAP)) that correlates closely with plasma

level of active metabolite. In addition, the dose/concentration-response is maintained without evidence for tolerance or tachyphylaxis over time. Differences in drug half-life may result in drug accumulation and more pronounced platelet inhibition during chronic therapy depending on the dose-interval employed. The pharmacokinetic and pharmacodynamic response to most oral GPIIb/IIIa inhibitors can be illustrated by comparing the contrasting responses of short-acting (ximelofiban, half-life 4.1 h) and longer-acting (sibrafiban and orbofiban; half-lives approximately 10–11 h) agents on roxifiban (18–hours).

Table 10.3
Clinical trials of oral GPIIb/IIIa inhibitors.

Xemilofiban

The first clinical trials of oral GPIIb/IIIa inhibition were with xerilofiban.^{23,35,45} As shown in Figure 10.1, platelet inhibition was achieved in a dose-dependent fashion with this oral GPIIb/IIIa inhibitor. It had a relatively short half-life and thus is given three times daily. The drug was well tolerated by patients in this study.

The Oral Glycoprotein IIb/IIIa Receptor Blockade to Inhibit Thrombosis (ORBIT) trial was a randomized dose-ranging trial of xemilofiban in patients undergoing percuta-

neous intervention.⁴⁶ Peak inhibition of platelet aggregation was similar following the same dose of xemilofiban administered on Days 14 and 28 of the trial. The time to peak blood level following the same dose of xemilofiban was reduced from 4 h following the first dose of drug to 2 h with steady-state dosing during chronic therapy.⁴⁶

The incidence and severity of bleeding events with 2 and 4 weeks of therapy by pharmacologic treatment regimen in the ORBIT trial is shown in Table 10.4. Most bleeding events were observed during the first 2 weeks

dromes was the OPUS-TIMI 16 trial, the preliminary results of which were presented at the American College of Cardiology in March 1999. This trial involved 10 302 patients randomized at 888 hospitals in 28 countries worldwide. The inclusion criteria were: onset within the last 72 hours of an acute coronary syndrome defined as rest ischemic pain lasting at least 5 min associated with either ECG changes, positive cardiac enzymes, or a prior history of vascular disease. Major exclusion criteria included renal insufficiency (creatinine >1.6 mg/dl or an estimated creatinine clearance of <40 cc/min, increased bleeding risk, or need for warfarin.

Eligible patients were treated with 150–162 mg of ASA, and were randomized, in double-blind fashion, to one of two dosing strategies of orbofiban given twice daily, or placebo. In one dose orbofiban was given 50 mg twice daily throughout the trial (50/50 group), in the other, the 50 mg twice daily dose was given for the first 30 days (the highest risk period), and then the dose was reduced to 30 mg twice daily (50/30 group). Other medical and interventional therapy was at the discretion of the treating physician. Patients are seen at 14 and 30 days and every 3 months. The primary endpoint was a composite of death, MI, recurrent ischemia leading to rehospitalization or urgent revascularization, or stroke. The planned sample size was to be 12 000 patients, but the trial was stopped prematurely after an unexpected finding of increased mortality at 30 days was observed in one of the orbofiban groups.

The preliminary findings on interim data showed: composite endpoint rates at 30 days were 10.7% in the placebo group vs. 9.5% in the two orbofiban groups ($p = 0.05$). Mortality at 30 days was 1.4% in the placebo group vs. 2.3% in the 50/30 group and 1.6% in the 50/50 group. Through follow-up, 300-day event rates were: 20.5, 20.2 and 19.5% respectively ($p = \text{NS}$). The safety profile was

acceptable, with the rate of major hemorrhage and thrombocytopenia within the expected range for this class of drugs. Subsequent exploratory analyses found greater benefit in patients who underwent percutaneous coronary intervention while on study drug and those who were stable on admission (Killip class I).

Data from the EXCITE trial of the agent xemilofiban in patients undergoing percutaneous coronary intervention were also presented, with similar results. No significant benefit was observed at 6 months.

Many lessons were learned from OPUS-TIMI 16, the first large trial of oral IIb/IIIa inhibition in acute coronary syndromes, which will be helpful in planning future trials of other IIb/IIIa inhibitors. First, it appears that it will be beneficial to optimize the dosing strategy used with the oral agents, potentially to mimic the stable antiplatelet effect achieved by the intravenous drugs. This would mean trying to reduce the inter- and intra-patient variability, potentially adjusting the dose by weight and/or renal function, as has been done in the SYMPHONY trials. One might also use plasma drug level and/or bedside platelet function test to adjust the dose. Second, our data suggest that one could target stabilize patients. In addition, several new and planned trials will be testing different drugs (e.g. with tight IIb/IIIa receptor binding), hence the field is moving forward.

Conclusions

Oral GPIIb/IIIa inhibitors may represent a major advance in the treatment of acute coronary syndromes, percutaneous coronary interventions, and stroke. They may also play a role not only in early treatment but also in secondary prevention. In early treatment, they may either be a substitute for, or a follow-up

to, intravenous compounds. To date, data are available only on the pharmacokinetic and pharmacodynamic effects of these agents. Numerous questions remain, such as what level of platelet inhibition is optimal, how efficacy and safety can best be balanced, whether

other adjunctive agents are needed, and whether monitoring of platelet function will assist in the use of these agents. Ongoing large-scale clinical trials will assess many of these issues and the clinical effects of this promising class of agents.

Miscellaneous antiplatelet agents: A physiologically based overview of platelet antagonist therapy

Richard C Becker

Introduction

The development of pharmacologic agents that offer platelet antagonist capabilities is a direct extension of our understanding of normal physiology and the laws that govern cellular events in the circulatory system. Under normal conditions, platelets circulate freely within the vasculature in a non-stimulated state and, as a result, little meaningful interaction takes place with other platelets, leukocytes or the vessel wall. It has become increasingly evident, however, that many of the recognized risk factors for atherosclerosis, and certainly atherosclerosis itself, have a profound impact on platelets, in essence, 'priming' them for future cell-cell and cell-vessel wall encounters. In the presence of advanced endothelial cell dysfunction, disruption or atheromatous plaque rupture, a complex chain of events is rapidly initiated, leading to platelet-rich thrombus formation. The responsible biochemical and cellular processes can be divided conceptually into five general categories:

- (1) Platelet adhesion;
- (2) Activation;
- (3) Secretion;
- (4) Aggregation; and
- (5) Support of coagulation.

Platelet adhesion

Platelets quickly and efficiently recognize abnormalities within the vascular system and adhere by means of adhesive proteins that interact with specific platelet membrane glycoproteins (receptors). To date, nine of the predominant and physiological important platelet membrane glycoproteins have been characterized.¹⁻⁴ The most common nomenclature for identification is based on polyacrylamide gel separation (Table 11.1, Figure 11.1). Most platelet membrane receptors consist of non-covalent complexes of individual glycoproteins or heterodimers (integrins) derived from α and β subunits. Platelets express at least two β subunits (β_1 and β_2) and five α subunits, which in varying combinations, identify distinct surface receptors.⁵

The initial events in adhesion are contact and binding, accomplished predominately by an interaction between the platelet glycoprotein Ib-IX complex and vonWillebrand factor.⁶ Other ligand-receptor interactions typically play a supportive role.

Platelet activation

Platelet activation can be triggered by a wide variety of biochemical and mechanical stimuli (in addition to platelet adhesion). Many of the biochemical agonists are produced or released by platelets themselves after vessel wall adhesion,

Receptor	Ligand	Integrin components	Biologic function
GPIa/IIa	Collagen	$\alpha_2\beta_1$	Adhesion
GPIb/IX	von Willebrand factor	—	Adhesion
GPIc/IIa	Fibronectin	$\alpha_5\beta_1$	Adhesion
GPIIb/IIIa	Collagen	$\alpha_{IIb}\beta_3$	Aggregation
	Fibrinogen		(secondary role in adhesion under high shear stress)
	Fibronectin		
	Vitronectin		
	von Willebrand factor		
GPIV (GPIIb)	Thrombospondin	—	Adhesion
Vitronectin	Collagen	$\alpha_v\beta_3$	Adhesion
VLA 6	Laminin	$\alpha_6\beta_1$	Adhesion

* GP, glycoproteins.

Table 11.1
Platelet surface membrane glycoproteins.*

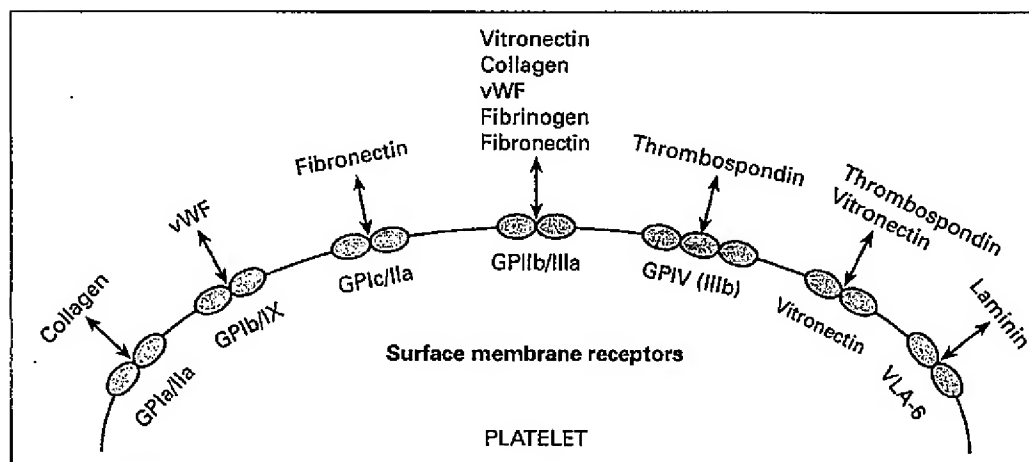


Figure 11.1
Diagram showing platelet surface membrane interactions. Properties inherent to platelets, including adhesion, activation, and aggregation are governed by membrane glycoproteins (receptors) that recognize one or more proteins (ligands). GP, glycoprotein; VFW, vonWillebrand factor.

initiating a positive feedback loop that amplifies the response to a given stimulus. The list of biochemical agonists is extensive, numbering 100 or more. The most physiologically relevant agonists are outlined in Table 11.2.

Platelet agonists bind surface glycoprotein receptors and stimulate signal transmission across the membrane via a messenger protein that, in turn, triggers one of two intracellular pathways. The phosphoinositide pathway is initiated by activation of phospholipase C. Phosphatidylinositol 4-5-bisphosphate (PIP₂) is cleaved to form two secondary messengers, inositol 1,4,5 triphosphate (IP₃) and diacylglycerol.⁷ IP₃ stimulates calcium mobilization from the dense tubular system. Increased cellular Ca²⁺ concentrations are required for activation of other intracellular enzymes responsible for physiologic platelet responses.⁸ Diacylglycerol activates protein C, causing protein phosphorylation, granule secretion and fibrinogen receptor expression.

The second pathway (phosphatidylcholine)

that can be initiated following platelet activation involves phospholipase A₂, which liberates arachidonate from cell membranes. Arachidonate is subsequently converted to thromboxane A₂ (TXA₂) by the platelet's cyclo-oxygenase enzyme system. TXA₂ is a potent platelet agonist in its own right, thus providing yet another positive-feedback mechanism that promotes the thrombotic mechanism (Figure 11.2). The platelet response to activation is summarized as follows:

- (1) Conformational change of GPIIb/IIIa (ligand and receptive);
- (2) Pseudopod formation and platelet shape change;
- (3) Surface expression of α -granule proteins (e.g. thrombospondin, fibrinogen);
- (4) Surface expression of granule membrane protein (P-selectin, GMP-140, CD62);
- (5) Development of coagulant activity through inside-out movement of membrane phospholipids;

Agonist	Source	Receptor(s)
Thrombin	Enzymatic end product of coagulation cascade	High-affinity (GPIIb) receptor
ADP	Platelet dense body, erythrocytes	ADP/aggrecin
Collagen	Subendothelial matrix	GPIa/IIa GPIIb/IIIa GPIV
Serotonin	Platelet dense body	5HT ₂ receptor
Thromboxane A ₂	Platelet membrane	PGH ₂ /TXA ₂ receptor
Platelet activating factor (PAF)	—	PAF receptor

* PAF, platelet activating factor; prostaglandin H₂; TXA₂, thromboxane A₂; GP, glycoprotein; ADP, adenosine diphosphate.

Table 11.2
Predominant biochemical agonists for platelet activation.*

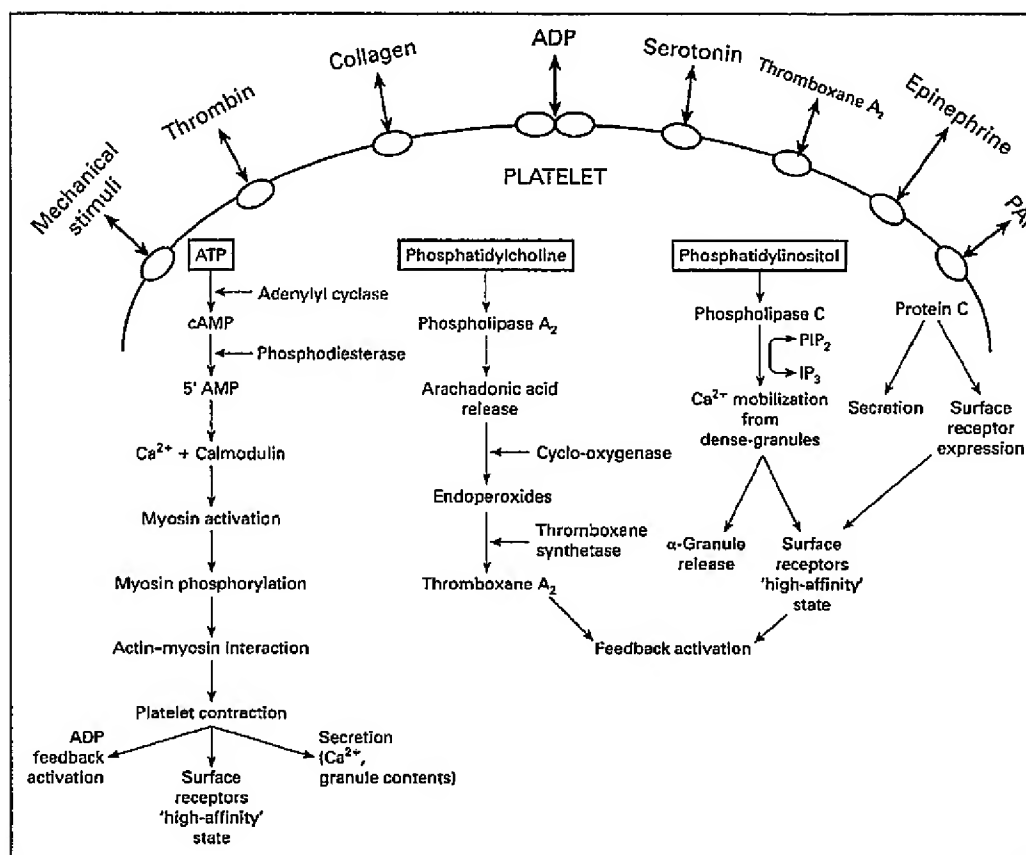


Figure 11.2

Platelet activation. This important process is triggered by a variety of biochemical and mechanical stimuli that provoke a series of internal events following their initial binding to specific surface receptors. The phosphoinositide and phosphatidylcholine pathways ultimately cause the release of calcium and physiologic agonists that stimulate further activation and potentiate aggregation through surface receptor expression. ADP, adenosine diphosphate; PAF, platelet activating factor; PIP₂, phosphatidylinositol 4-5-biphosphate; IP₃, inositol 1,4,5 triphosphate.

- (6) Expression of coagulation proteins (e.g. factor V);
- (7) Secretion of α and dense-granule contents;
- (8) Increased cytosolic Ca²⁺;
- (9) Activation of phospholipase C;
- (10) Mobilization of Ca²⁺;
- (11) Activation of phospholipase A₂; and
- (12) Activation of protein kinase C.

Platelet agonists can be classified as strong or weak. Strong agonists, for example, thrombin affect both phosphoinositide hydrolysis and arachidonate metabolism (via phospholipase C and phospholipase A₂). Accordingly, their ability to promote platelet activation and aggregation persists despite inhibition of one of the two pathways. Indeed, it has been shown that even low concentrations of thrombin (≤ 0.1 U/ml) can produce platelet aggregation in the face of inhibition of platelet TXA₂ production.⁹ Weak agonists (collagen and adenosine diphosphate, for example) lack the ability to trigger phosphoinositide hydrolysis and are more dependent on TXA₂ formation for their effects. Studies dating back several decades revealed that inhibition of TXA₂ formation could reduce collagen-induced platelet aggregation.¹⁰

Platelet secretion

Platelet activation prompts the secretion of contents from within three different types of platelet storage granules: lysosomes, α -granules, and dense bodies. The exact mechanism of granule secretion is largely undetermined but it is felt to involve an energy-dependent contractile process, resulting in extrusion of granule contents. Fusion of α -granules with each other and with deep invaginations of the plasma membrane (the open canalicular system) followed by an 'emptying' of contents to the exterior has since been demonstrated.^{11,12} It is unclear if other platelet granules use a similar mechanism to release their contents.

Platelet aggregation

Platelet aggregation is considered the physiologic goal of platelet activation because it is through platelet aggregation that primary hemostasis can occur. As already reviewed, a variety of agonists can stimulate platelets via interaction with specific membrane receptors,

followed by production of secondary messengers, which in turn promote a series of intracellular events. One of the most important platelet responses triggered is a conformational change in the glycoprotein (GP)IIb/IIIa membrane receptor that facilitates an interaction between fibrinogen and its receptor and thus forming multiple cross-links between adjacent platelets. This reaction represents the 'final common pathway' for platelet activation and is a vital process in the formation of platelet-rich thrombi. Accordingly, investigators have focused their attention on this fundamental event in attempting to develop new platelet antagonists for clinical use.

Platelet support of coagulation

The phospholipid membrane of activated platelets and of platelet aggregates forms an ideal template for coagulation processes that facilitate thrombus growth (a second-wave phenomenon). The prothrombinase complex, responsible for the conversion of prothrombin to thrombin, consists of factor V₃ (provided by activated platelets), factor X₃, phospholipid and calcium. Thrombin, in turn, converts fibrinogen to fibrin that is responsible for stabilization of the platelet-rich thrombus (Figure 11.3). It is very important to recognize that, although platelets are the predominant source of phospholipid in both physiologic hemostasis and pathologic thrombosis, prothrombinase assembly can occur on dysfunctional vascular endothelial cells and factor X₃ can be generated through tissue factor that is present in high concentrations within atheromatous plaques and on the surface of activated monocytes.^{13,14}

Platelet and vessel wall physiology and pharmacologic interventions

An understanding of platelet behavior provides the cornerstone of pharmacologic

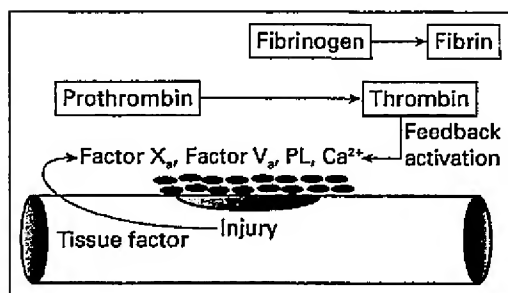


Figure 11.3

Platelet aggregates at a site of vessel wall injury. These serve as a template for assembly and activation of the prothrombinase complex (factor X, factor V, phospholipid (PL) and calcium), which rapidly converts prothrombin to thrombin. Thrombin, a pivotal enzyme, converts fibrinogen to fibrin and also stimulates autocatalytic activation of factors V, VIII (tenase complex) and X. The prothrombinase complex also can be assembled on dysfunctional vascular endothelial cells and atheromatous plaques where tissue factor serves as a potent stimulus.

approaches to the treatment of patients with disorders characterized by enhanced platelet adhesion, activation, aggregation and/or support of coagulation (prothrombinase assembly and activity).

A summary of agents affecting platelet and vessel wall physiology are summarized in the following list.

(1) *Agents that inhibit platelet adhesion*

- von Willebrand factor monoclonal antibodies
- Aurintricarboxylic acid
- GPIIb/IIIa receptor antagonists (high shear stress)

(2) *Agents that inhibit platelet activation*

- Prostacyclin
- Prostaglandin E₁
- Prostanoid analogs (iloprost, beraprost, cicaprost, ciprostone)
- Thromboxane/endoperoxide receptor antagonists
- Platelet activating factor antagonists

(3) *Agents that inhibit platelet aggregation*

- P_{2T} purinoceptor antagonists
- Nitric oxide/nitric oxide donors
- Apyrase
- GPIIb/IIIa receptor antagonists
- Aspirin
- Non-steroidal anti-inflammatory agents (NSAIDs)
- Dipyridamole
- Ticlopidine
- Clopidogrel
- Dextran
- Omega-3 fatty acids
- Cilostazol
- Ketanserin
- Ridogrel
- Angiotensin converting-enzyme inhibitors
- Vitamin E

(4) *Agents that inhibit platelet secretion*

- Calcium-channel antagonists

(5) *Agents that inhibit prothrombinase assembly on platelet surface*

- Low-molecular-weight heparins
- GPIIb/IIIa antagonists

Platelet antagonists

Agents that inhibit platelet adhesion

The adhesion of platelets to a site of vessel wall injury is mediated by von Willebrand factor

tor that binds to the platelet GPIb/IX complex receptor (and the GPIIb/IIIa receptor under high shear stress conditions). Monoclonal antibodies to vonWillebrand factor have been developed and tested in animal models,¹⁵ as has aurintricarboxylic acid,¹⁶ which is a triphenylmethyl compound that inhibits vonWillebrand factor binding. To date, investigation in humans has not taken place, perhaps because of concerns regarding the potential risk for hemorrhagic complications.

Although the GPIIb/IIIa receptor antagonists are best known for their ability to inhibit platelet aggregation (discussed in detail in Chapters 8, 9 and 10), under high shear stress conditions vonWillebrand factor can also bind the GPIIb/IIIa receptor, facilitating adhesion. As a result, GPIIb/IIIa antagonists may have an impact on both platelet adhesion and aggregation.

Agents that inhibit platelet activation

As previously discussed, platelet activation is followed by a series of intracellular events that culminate in the release of calcium and substances that augment platelet aggregation and support of the coagulation cascade. Thus, pharmacologic agents that inhibit initial surface receptor-mediated activation also impair platelet aggregation.

Prostaglandin E and prostacyclin

Several natural prostanoids (PGE₁ and PGI₂) can inhibit platelet activation and aggregation by elevating cyclic AMP (cAMP) levels. Although the mechanism is complex, the primary mode of inhibition is through the activation of adenylate cyclase (with a subsequent rise in cAMP concentrations), that in turn, prevents calcium mobilization. The clinical application of PGE₁ and PGI₂ has been limited by their effect on vascular tone, producing

substantial systemic hypotension,¹⁷⁻¹⁹ and by extensive first-pass metabolism in the lungs (70% of the active compound is rapidly cleared).^{20,21}

The prostanoid analogs (e.g. iloprost, beraprost, cicaprost, ciprostone) are more stable compounds than PGE₁ and PGI₂; however, their development has focused primarily on potential use in patients with primary pulmonary hypertension.²²

Thromboxane/endoperoxide receptor antagonists

This class of compounds is designed to prevent platelet activation in response to thromboxane A₂ and other endoperoxides. There is a limited experience with the thromboxane receptor antagonists sulotaban and SQ30741, in patients with myocardial infarction treated with streptokinase²³ and tPA,²⁴ respectively. Ridogrel, a thromboxane synthetase antagonist, that also has antagonistic effects on the thromboxane receptor, was shown to reduce recurrent ischemia compared with aspirin when used adjunctively with thrombolytic therapy;²⁵ however, further investigation on a large scale has not yet taken place.

Agents that inhibit platelet aggregation

Serotonin receptor antagonists

Ketanserin, a serotonin receptor antagonist, has been studied in animal models of coronary thrombosis and thrombolysis where it has been shown, when administered concomitantly with a thromboxane A₂ receptor antagonist, to improve reperfusion and decrease reocclusion following tPA administration.²⁶

Aspirin and platelet antagonists (see also Chapter 6)

Aspirin acetylates platelet cyclo-oxygenase and impairs prostaglandin metabolism and

thromboxane A_2 synthesis, is discussed in detail within Chapter 6. The potent class of platelet antagonists that prevent the binding of fibrinogen to its GPIIb/IIIa receptor (GPIIb/IIIa receptor antagonists) on the platelet surface is discussed in Chapter 9.

Thromboxane synthetase inhibitors

Thromboxane synthetase antagonists, including dazoxiben and piroxicam, suppress platelet thromboxane synthesis and platelet aggregation.^{41,42} Clinical development has been hampered by the aggregating potential of endoperoxide intermediates and by the incomplete inhibition of thromboxane synthesis by currently available compounds.

Dextran

Dextran is a polysaccharide preparation that ranges in molecular weight from 65–80 kD. It prolongs the bleeding time, probably by interfering with surface membrane receptor function and fibrinogen binding.⁴³ Dextran also reduces plasma viscosity.

Omega-3 fatty acids

Omega-3 fatty acids decrease platelet membrane arachidonic acid concentration, reducing thromboxane A_2 synthesis. The competition of N-3 polyunsaturated fatty acids for cyclo-oxygenase also reduces platelet aggregating capacity by facilitating the synthesis of biologically inactive prostanoids.^{44,45}

Nitric oxide

Nitric oxide (NO) is a naturally occurring molecule derived from the amino acid L-arginine. It is a product of normal vascular endothelial cells and plays a critical role in maintaining both vasoreactivity and thromboresistance.

NO prevents platelet adhesion and also inhibits agonist-dependent G-protein-mediated

phospholipase C activation with subsequent calcium release. Accordingly, NO prevents P-selectin expression and calcium-dependent conformation change in platelet surface GPIIb/IIIa. It has also been shown to potentiate platelet disaggregation by preventing the stabilization of fibrinogen-GPIIb/IIIa interactions.⁴⁶ Beyond having potent platelet inhibitory effects, NO also inhibits neutrophil aggregation in vitro and prevents leukocyte adhesion to vascular endothelium.

Although the endothelial cell is a major source of NO, it is not the sole source. Platelets themselves, and their precursors megakaryocytes, possess NO synthase activity.⁴⁷ Vascular endothelial cells produce NO at a basal rate that can be augmented in response to physiologic stimuli including platelet release products, thrombin, shear stress and changes in oxygen tension.

Organic nitrates and other nitrovasodilators serve as an exogenous source of NO. Because nitroglycerin and nitroprusside have platelet inhibitory effects and promote platelet disaggregation in vitro.⁴⁸ The mechanism by which organic nitrates release NO remains controversial but it seems most likely that the former are converted to bioactive NO by a surface enzyme system.⁴⁹ In a double-blind randomized, placebo controlled trial, hypercholesterolemic patients were assigned to L-arginine hydrochloride (8.4 g/day orally or placebo) for 2 weeks. Platelet aggregation in response to collagen (5 µg/ml), was increased at baseline in patients with a marked reduction following treatment. The effect lasted for 2 weeks after completion of the treatment phase.⁵⁰ L-Arginine has been shown to reduce human monocyte adhesion to endothelial cells and may also decrease the expression of several cellular adhesion molecules.⁵¹

In addition to their direct effects, organic nitrates undergo denitrication with formation

tion of S-nitrosothiol (RSNO) intermediates. These species inhibit platelet aggregation through cyclic GMP (cGMP).⁵² A poly-nitrosated RSNO, S-nitroso-BSA, administered locally following femoral artery injury in a rabbit model, prevented neointimal proliferation and platelet adhesion.⁵³ N-acetyl-L-cysteine enhanced the platelet inhibitory effects of nitroglycerin⁵⁴ and S-nitroso-N-acetyl-L-cysteine decreased platelet function by reducing the expression of ligand receptor GPIIb/IIIa.⁵⁵ In patients with acute coronary syndromes S-nitrosoglutathione reduced platelet activation and GPIIb/IIIa expression.⁵⁶

RSNOs have been used in animal models to prevent leukocyte-mediated tissue damage and reperfusion injury. In a rat splanchnic artery model of ischemia, the NO donor, S-nitroso-N-acetyl-D,L penicillamine caused reduced leukocyte-endothelial cell interactions.⁵⁷ S-Nitrosated tissue plasminogen activator reduced myocardial necrosis and preserved endothelial function in a feline model of ischemia and reperfusion.⁵⁸

NONOates

Complexes of nitric oxide with nucleophiles, known as NONOates, are capable of spontaneously generating NO and, as a result, may offer therapeutic benefit in the treatment of NO deficiency states. The biologic potency, as well as duration of action, can be modified by altering the carrier nucleophile. For example, DEA (diethylamine)/NO possesses a shorter half-life than SPER (spermine)/NO (2.1 min versus 39 min, respectively) resulting in an earlier peak activity (5 min versus 15 min) and a shorter duration of action. In contrast to the acid stability of RSNOs, NONOates are alkali stable and decompose rapidly at low pH.⁵⁹

DEA/NO and SPER/NO have been shown to have potent antiplatelet properties. Platelet aggregation measured in whole blood or

platelet-rich plasma (PRP) following the addition of collagen was reduced by DEA/NO in a dose-dependent manner. The effect was similar to aspirin in whole blood. In vivo both agents demonstrated antiplatelet activity that correlated with the rate of release of NO in solution.⁶⁰

A rapid NO donor, PROLI/NO, formed by the reaction of nitric acid with L-proline in methanolic sodium methoxide, dissociates to proline (1 mole) and NO (2 moles) with a half-life of 1.8 s at a pH 7.4 (37°C) and possesses both antiplatelet and vasodilatory properties. When infused into an unheparinized polyester vascular graft (baboon model) platelet deposition was reduced significantly.⁶¹

Recently, the NONOate group has been incorporated into polymeric matrices that can be applied onto therapeutic surfaces such as vascular grafts. Platelet function in vivo has been evaluated in a baboon artery-to-vein shunt coated with a polymer containing the NONOate functional group. When compared with uncoated grafts, the NO treated grafts were found to be less thrombogenic.⁶²

Molsidomine and SIN-1

A novel class of nitrosovasodilators, the sydnonimines, that include molsidomine and its active metabolite SIN-1, has been evaluated clinically as effective NO donors. SIN-1 reacts with molecular oxygen resulting in the spontaneous release of NO through a process that involves a 1-electron abstraction.⁶³

It has been suggested that administration of molsidomine and SIN-1 may decrease mortality associated with acute myocardial infarction by up to 35%. To confirm this observation, the ESPRIM (European Study of Prevention of Infarct with Molsidomine) trial randomized 4017 patients with acute myocardial infarction to receive either SIN-1 (1 mg/h intravenously for 48 h) followed by molsidomine (16 mg orally for 12 days) or placebo.

Although there was no difference in all-cause mortality between groups at either 35 days or 13 months; the study was considered inconclusive, based on the inclusion of predominantly low-risk patients.⁶⁴

An oral extended release preparation of molsidomine was evaluated in a small randomized trial of 50 patients with known ischemic heart disease and chronic stable angina to determine its effect on both symptoms and overall exercise capacity. Patients received either study drug or placebo and underwent exercise testing at baseline, 2, 4, 6, 8 and 10 h. Exercise duration and performance were enhanced and ST-segment depression, a marker of ischemia, was reduced up to 10 h following administration of molsidomine. Further, anginal attacks and sublingual nitrate use were reduced in the treatment group.⁶⁵

Molsidomine and SIN-1, as NO donors, inhibit vascular smooth muscle cell proliferation. Therefore, it has been suggested that these agents may reduce the occurrence of restenosis following percutaneous coronary interventions. The ACCORD (Angioplastic Coronaire Corvasal Diltiazem) study evaluated 700 stable patients scheduled for coronary angioplasty and randomized them to receive either SIN-1 or diltiazem. Therapy was administered prior to coronary intervention and continued for 6 months afterward. Patients receiving SIN-1 demonstrated a greater luminal diameter pre- and post-angioplasty, as well as at 6-month angiographic follow-up. Although restenosis rates were significantly reduced in the SIN-1 group, this effect did not translate into a difference in combined clinical events, including death and non-fatal MI.⁶⁶

Pirsidomine

Pirsidomine, *N-p*-anisoyl-3-(*cis*-2,6-dimethylpiperidino) sydnonimine, possesses hemody-

namic properties similar to molsidomine, but has a longer duration of action. In animals subjected to coronary arterial occlusion pirsidomine administration reduced the occurrence of ventricular ectopy and delayed the time to onset of ventricular fibrillation. Leukocytes recovered from animals treated with pirsidomine, generated less superoxide determined by lumino-enhanced chemiluminescence than those not treated.⁶⁷

New NO donors

Several novel NO donors are currently under development. The compound FK 409, (\pm)(E 4-ethyl-3[(Z)-hydroxyimino]-5-nitro-3-glycyloxy)-3-pyridinecarboxamide, similarly releases NO but at a slower rate. When compared with FR 144420 in an isolated rat aortic preparation, FK 409 demonstrated greater vasorelaxant potency and hemodynamic effect although its duration of action was shorter than that seen with FR 144420.⁶⁸

IFT 296

The nitrate ester IFT 296, [3-(2-nitrooxyethyl)-3,4-dihydro-2H-1,3-benzoxazin-4-one] has demonstrated anti-ischemic effects in an isolated rabbit heart model subjected to global ischemia.⁶⁹

SPM-5185

The compound SPM-5185, (N-nitratopivaloyl)-S-(N'-acetylalanyl)-cysteine ethylester, an effective NO donor. SPM-5185 was compared to nitroglycerin in an ex vivo preparation of human saphenous vein grafts at the time of bypass grafting. SPM-5185 produced comparable relaxation in both arteries and veins, was less prone to the development of tolerance, and effectively produced vasorelaxation in vessels that developed tolerance to nitroglycerin.⁷⁰

Agents that inhibit platelet-dependent prothrombinase assembly and activity

Anticoagulants

The platelet surface serves as a pivotal site for assembly of the procoagulant intrinsic 'tenase' complex, that leads to factor X activation, and prothrombinase. Thus, platelet activation can be viewed as a thrombin-generating system that contributes to a rapid increase in local concentrations of thrombin, as well as a persistent site of thrombin generation.

In a recent series of experiments, platelet activation in response to ADP was greatest in blood anticoagulated with unfractionated heparin compared with hirudin, recombinant tick anticoagulant peptide or enoxaparin (a low-molecular-weight heparin preparation).⁷¹ The effect may have been driven by the negative charge of unfractionated heparin and its tendency to bind thrombospondin, a platelet α -granule adhesive protein, and platelet factor

4, increasing platelet activation in response to biochemical mediators.^{72,73} Although the physiologic implications of this observation are yet to be determined, they suggest that platelet activation may be facilitated by unfractionated heparin and attenuated by factor Xa and/or direct thrombin antagonists.

The ability of low-molecular-weight heparin to inhibit platelet-dependent prothrombinase assembly and activity was investigated by Spencer and colleagues.⁷⁴ Samples were obtained from patients with a presenting diagnosis of unstable angina or non-ST segment elevation MI who had received enoxaparin (Lovenox®). Using samples obtained 1 h after enoxaparin administration (peak concentration), platelet-dependent prothrombinase activity (thrombin generation represented by prothrombin fragment 1.2) was reduced by approximately 25% compared with baseline (pre-treatment) (Figure 11.4). A similar reduction in prothrombinase activity was observed using samples obtained 24–48 h after the

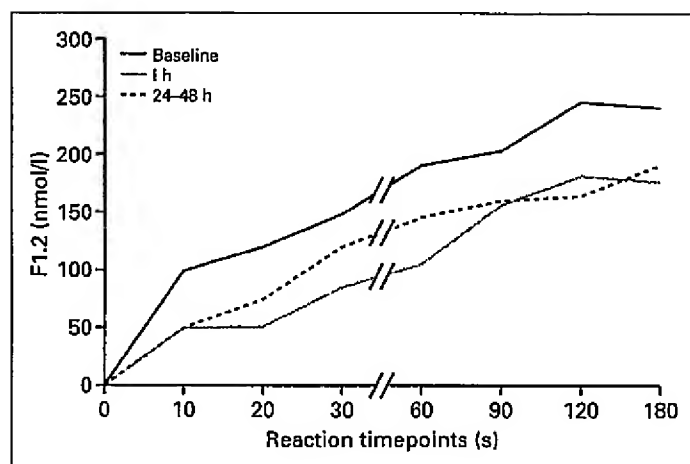


Figure 11.4
Prothrombinase inactivation. This was achieved using the low-molecular-weight heparin preparation, enoxaparin, among patients with unstable angina and non-ST segment elevation MI. Inactivation, as determined by prothrombin fragment 1.2 (F1.2) generation, was greatest with higher plasma concentrations (1 h after a 30 mg intravenous bolus) but was also observed at steady-state concentration (24–48 h on a maintenance dose of 1.0-mg/kg sc twice daily).

initiation of treatment (steady-state enoxaparin concentrations). In a separate series of experiments, samples from patients receiving enoxaparin reduced tissue factor-mediated prothrombinase assembly (and subsequent thrombin generation) (Figure 11.5).

The findings of Spencer and colleagues suggest that enoxaparin is able to inactivate platelet prothrombinase as well as inhibit tissue factor-mediated prothrombinase assembly. Both properties, which may have important implications for the treatment of patients with acute coronary syndromes, could be explained by the ability of enoxaparin to inactivate platelet bound factor X_a . It follows that more potent and specific X_a inhibitors may offer considerable promise in the management of arterial thrombotic disorders.

Platelet antagonists

If platelets contribute substantially to thrombin generation in vivo, it is possible that

platelet antagonists themselves can decrease thrombin generation. Decreased platelet deposition, activation and aggregation would reduce the template for thrombin generation and fibrin formation, yielding a less stable thrombus.

Inhibitors of GPIIb/IIIa have received considerable attention, not only as potent platelet antagonists but also as anticoagulants. Initial support for the latter was derived from a large scale clinical trial (EPIC) in which patients treated with abciximab (ReoPro®) and heparin had longer activated clotting times (ACT) than those receiving heparin alone at the time of coronary interventions.⁷⁴ Work in our laboratory supports the ability of other GPIIb/IIIa antagonists to inhibit thrombin generation as well. In vitro experiments with the selective non-peptide tirofiban identified a dose dependent inhibition of tissue factor mediated thrombin generation (Figure 11.6). Although the mechanism for the anticoagulant effects

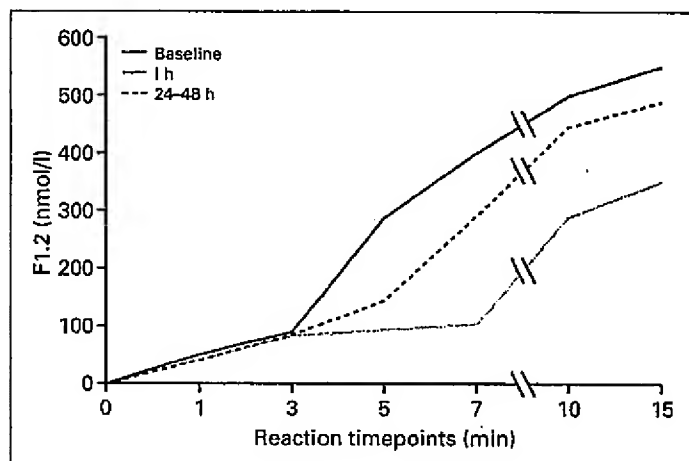
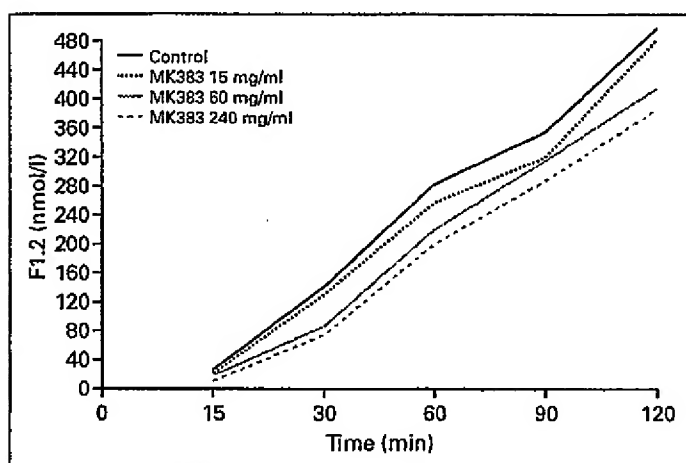


Figure 11.5
Inhibition of prothrombin formation and activity. Plasma samples were obtained from patients with unstable angina or non-ST-segment elevation MI receiving enoxaparin inhibited both prothrombinase formation and activity. Inhibition was greatest 1 hour after a 30 mg intravenous bolus. F1.2, prothrombin fragment F1.2.

**Figure 11.6**

Inhibition of tirofiban (MK383) on platelet coagulant activity in the presence of fibrinogen (3 mg/ml). The platelet GPIIb/IIIa surface receptor antagonist tirofiban, in addition to its ability to prevent platelet aggregation, inhibits thrombin generation (F1.2) in a dose-dependent manner. Plasma systems with washed platelets, thrombin, CaCl_2 , factor V_a , factor X_a , and fibrinogen.

unknown, it is possible that GPIIb/IIIa blockade impairs microparticle formation and prothrombinase assembly. Studies to define the antithrombotic potential of GPIIb/IIIa antagonists in greater detail are ongoing.

New developments in platelet inhibition

An ability to target specific receptors and intracellular signaling events that lead to pathologic thrombosis, offers considerable potential in clinical medicine.

The platelet inhibiting potential of ticlopidine and its derivative, clopidogrel, are well recognized and are discussed in detail in Chapter 7. In order to understand and appreciate the newest class of platelet inhibitors, purine receptor antagonists, some initial discussion of the ADP receptor is required.

The platelet ADP receptor (see also Chapter 7)

Adenosine diphosphate (ADP) was the first nucleotide to be identified in blood that

could account for changes in platelet behavior upon exposure to a foreign surface. In fact, ADP extracted from erythrocyte membranes was shown to increase the ability of platelets to stick to glass.^{27,28} Since that time, a wide variety of pharmacologic responses to nucleotides have been identified and a comprehensive classification of nucleotide receptors has been developed.²⁹⁻³²

The receptors, classified by their preference for a variety of nucleotide analogs as agonists are referred to as P_2 purinoceptors. This distinguishes them from receptors that recognize adenosine, which are known as P_1 purinoceptors. The P_2 purinoceptor includes three separate categories P_{2X} , P_{2Y} and P_{2T} , based on structural criteria and the order of cloning.

The P_2 receptor has two hydrophobic domains. To date, no specific competitive antagonists have been identified that distinguish between the P_{2X} and P_{2Y} receptors. The P_{2Y} receptor has seven hydrophobic domains and resembles the rhodopsin family of recep-

tors that interact with G-proteins to activate phospholipases, or to stimulate (or inhibit) adenylyl cyclase. Both ADP and ATP are agonists for the P_{2X} and P_{2Y} purinoceptors. In contrast, the P_{2T} receptor is activated by ADP, while it is inhibited (by competitive antagonism) by ATP. These unique properties have led to the development of ATP analogs with high potency and specificity for the P_{2T} receptor (discussed later).

Binding

The binding of [14 C] ADP to the platelet surface is achieved through a specific receptor site (molecular weight 61 kDa) with approxi-

mately 100 000 copies per cell (affinity constant $K = 6.5 \times 10^6 \text{ M}^{-1}$).³³ Competition: binding at the ADP receptor is as follows: $\text{ATP} = \text{ADP} > \text{AMP} \gg \text{adenosine}$.

Mechanisms of action

A variety of platelet responses have been reported following ADP binding to its receptor. These include rapid calcium influx, mobilization of intracellular calcium stores, shape change, inhibition of adenylyl cyclase, stimulation of IP_3 formation, expression of GPIIb/III phospholipase A_2 stimulation, release of dense-granule contents and release of α -granule contents (Figure 11.7).

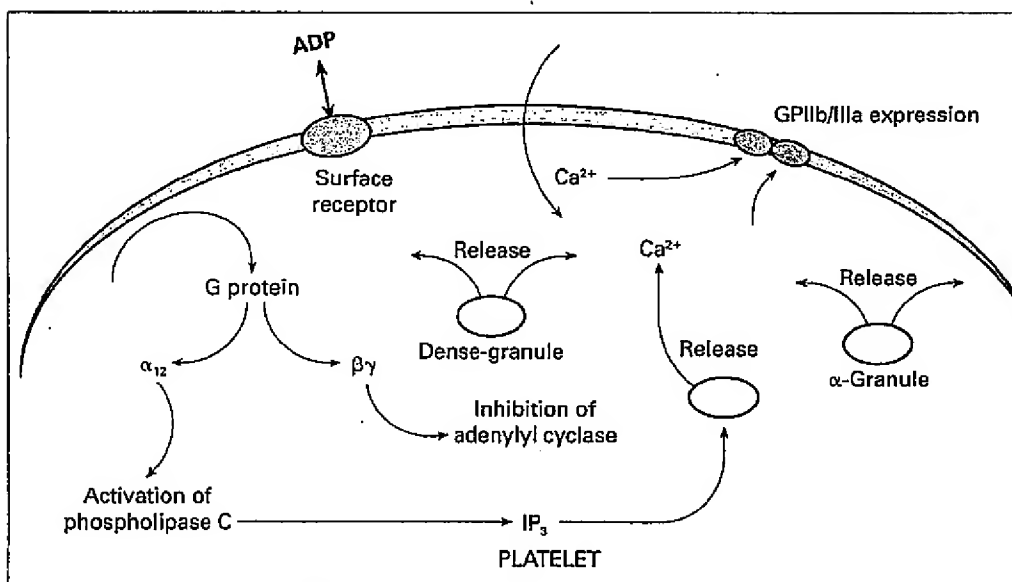


Figure 11.7

Adenosine diphosphate (ADP) binding to the platelet surface. This can happen via one or more membrane receptors. Following receptor stimulation, internal signaling takes place followed by dissociation of heterotrimeric G_i protein to α_{12} and $\beta\gamma$ subunits that activate phospholipase C and inhibit adenylyl cyclase, respectively. Ultimately, the release of agonists from both dense and alpha granules takes place and surface expression of GPIIb/IIIa is provoked.

ADP receptors on other cells

ADP receptors exist on cells other than platelets and this may have physiologic importance. ADP promotes the binding of fibrinogen to monocytes³⁴ and stimulates calcium mobilization in megakaryocytes. ADP receptors have also been identified on glioma cells, hepatocytes, and capillary endothelial cells.³⁵

 P_{2T} purinoceptor antagonists

Adenosine triphosphate (ATP) is a competitive P_{2T} purinoceptor antagonist that can inhibit ADP-mediated platelet aggregation. However, since ATP functions as an agonist at other P_2 receptor sites, efforts are underway to develop selective P_{2T} receptor antagonists that can be

used clinically in situations where platelet activation, aggregation, and platelet-rich thromboses are prevalent (e.g. in acute coronary syndromes).

A novel ATP analog, FPL66096 (2-propylthio-D-B₁4-difluoromethylene ATP) produces a dose-dependent inhibition of ADP-mediated platelet aggregation with a high degree of selectivity for the P_{2T} purinoceptor.³⁶ The dichloro-derivative molecule, FPL67085 is a potent ADP-mediated platelet antagonist as well, and has been shown to prevent cyclic flow variations in a Folts model.³⁷ Its antithrombotic effects were similar to those of GPIIb/IIIa antagonists but at much less prolongation in the bleeding time (Figure 11.8).³⁸

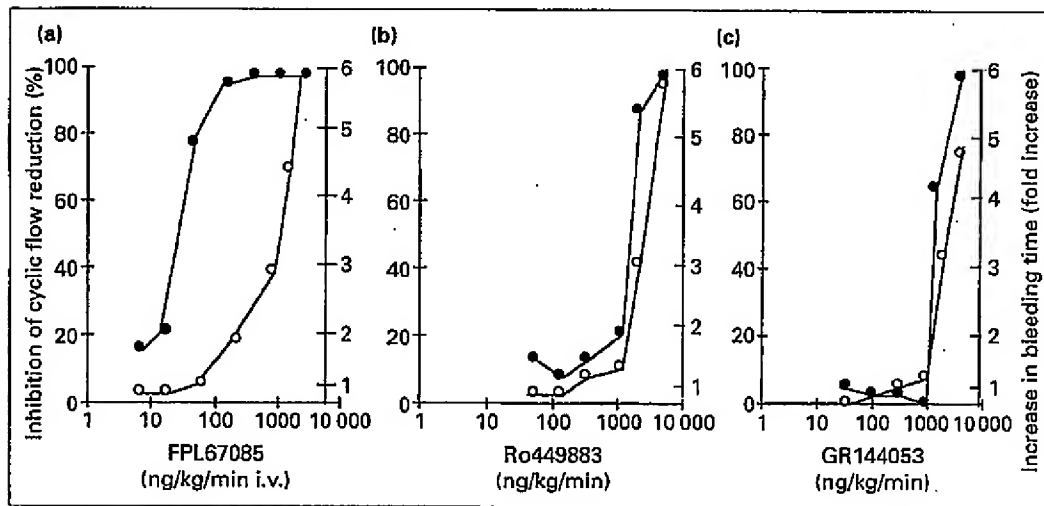


Figure 11.8

Comparison of the antithrombotic effect of (a) the P_{2T} purinoceptor antagonist FPL67085 and two GPIIb/IIIc receptor antagonists (b) RO449883 and (c) GR144053, in a canine model of coronary thrombosis (●, % inhibition of cyclic flow reduction), (○, bleeding time increase). From Humphries RG, 1995; with permission.³⁸

When compared with ticlopidine and aspirin in an anesthetized rat model³⁹ FPL 67085 was found to be a more effective inhibitor of ADP-mediated platelet aggregation.

A second novel ATP analog, AR-C69931 MX (2-methylthio-ethyl-2-3,3,3-trifluoropropyl (adenylic acid) is a potent inhibitor of ADP-induced aggregation in human washed platelets (in vitro). It has also been shown to prevent arterial thrombosis in a canine model with minimal prolongation of the bleeding

time. The latter observation has also been confirmed in studies of healthy human volunteers in whom platelet aggregation in response to ADP was eventually abolished at doses that prolonged the bleeding time by approximately 2-fold (Figure 11.9). ADP-mediated ex vivo aggregation returned to normal within 20 min of terminating the infusion.⁴⁰ The metabolism of AR-C69931 MX is predominately via the hepatic route with less than 10% being excreted through the kidneys.

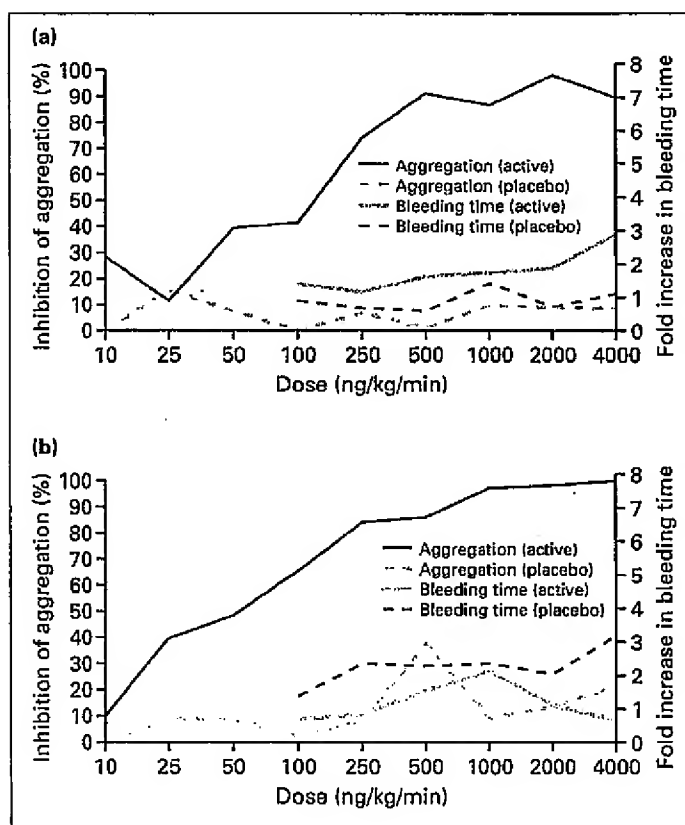
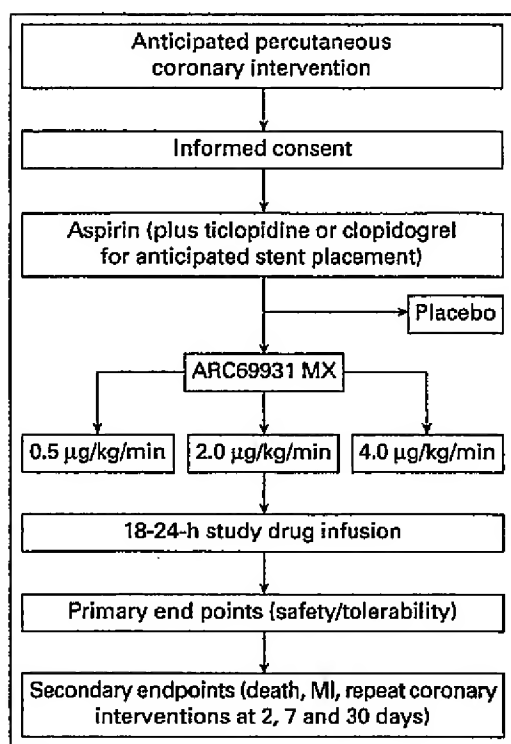


Figure 11.9
Platelet inhibition and bleeding time prolongation with increasing concentrations of the P_{2T} purinoceptor antagonist: (a) AR-C69931 MX, in healthy female subjects; (b) AR-C69931 MX, in healthy male subjects. Humphries RG, Personal communication.⁴⁰

**Figure 11.10**

Proposed study design for a Phase II clinical trial of ARC69931 MX in patients with coronary artery disease undergoing percutaneous coronary interventions.

Clinical experience

The specific P_{2T} receptor antagonist, AR C69931 MX, has been given to patients with unstable angina and non-ST segment elevation MI in phase II clinical trials. A double-blind, placebo-controlled multicenter dose ranging study of approximately 450 patients is being conducted in the USA to assess the safety and tolerability of intravenous AR C69931 MX (doses: 0.5 $\mu\text{g/kg/min}$, 2.0 $\mu\text{g/kg/min}$, 4.0 $\mu\text{g/kg/min}$) given for 18–24 h in patients undergoing percutaneous coronary interventions (Figure 11.10). Preliminary safety results are favorable and have stimulated further investigation.

Summary

A comprehensive and expanding knowledge of platelet cellular anatomy and physiology, coupled with an understanding of pathobiologic events that govern coronary arterial thrombosis in patients with acute ischemic syndromes has paved the way for pharmacologic advances in antithrombotic therapy. Although much investigation is needed, antagonists of the platelet ADP receptor and nitric oxide derivatives, either alone or administered conjunctively with a GPIIb/IIIa inhibitor, appear particularly attractive for development, and, if deemed worthy, clinical use.

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Exhibit 26

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Prasugrel versus Clopidogrel in Patients with Acute Coronary Syndromes

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ABSTRACT

BACKGROUND

Dual-antiplatelet therapy with aspirin and a thienopyridine is a cornerstone of treatment to prevent thrombotic complications of acute coronary syndromes and percutaneous coronary intervention.

METHODS

To compare prasugrel, a new thienopyridine, with clopidogrel, we randomly assigned 13,608 patients with moderate-to-high-risk acute coronary syndromes with scheduled percutaneous coronary intervention to receive prasugrel (a 60-mg loading dose and a 10-mg daily maintenance dose) or clopidogrel (a 300-mg loading dose and a 75-mg daily maintenance dose), for 6 to 15 months. The primary efficacy end point was death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke. The key safety end point was major bleeding.

RESULTS

The primary efficacy end point occurred in 12.1% of patients receiving clopidogrel and 9.9% of patients receiving prasugrel (hazard ratio for prasugrel vs. clopidogrel, 0.81; 95% confidence interval [CI], 0.73 to 0.90; $P < 0.001$). We also found significant reductions in the prasugrel group in the rates of myocardial infarction (9.7% for clopidogrel vs. 7.4% for prasugrel; $P < 0.001$), urgent target-vessel revascularization (3.7% vs. 2.5%; $P < 0.001$), and stent thrombosis (2.4% vs. 1.1%; $P < 0.001$). Major bleeding was observed in 2.4% of patients receiving prasugrel and in 1.8% of patients receiving clopidogrel (hazard ratio, 1.32; 95% CI, 1.03 to 1.68; $P = 0.03$). Also greater in the prasugrel group was the rate of life-threatening bleeding (1.4% vs. 0.9%; $P = 0.01$), including nonfatal bleeding (1.1% vs. 0.9%; hazard ratio, 1.25; $P = 0.23$) and fatal bleeding (0.4% vs. 0.1%; $P = 0.002$).

CONCLUSIONS

In patients with acute coronary syndromes with scheduled percutaneous coronary intervention, prasugrel therapy was associated with significantly reduced rates of ischemic events, including stent thrombosis, but with an increased risk of major bleeding, including fatal bleeding. Overall mortality did not differ significantly between treatment groups. (ClinicalTrials.gov number, NCT00097591.)

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THE SHORT-TERM AND LONG-TERM BENEFITS of dual-antiplatelet therapy with aspirin and clopidogrel have been established for patients with acute coronary syndromes¹⁻³ and those undergoing percutaneous coronary intervention (PCI).^{4,5} Despite these benefits, many patients continue to have recurrent atherothrombotic events while receiving standard dual antiplatelet therapy.¹ In addition, important limitations of clopidogrel remain, such as only a modest antiplatelet effect, with substantial interpatient variability^{6,7} and a delayed onset of action.⁵ Small clinical studies have suggested that patients with a reduced pharmacologic response to clopidogrel may be at increased risk for adverse clinical events, including myocardial infarction and coronary-stent thrombosis.⁸⁻¹¹

Prasugrel — a novel thienopyridine — is a pro-drug that, like clopidogrel, requires conversion to an active metabolite before binding to the platelet P2Y₁₂ receptor to confer antiplatelet activity.¹² At the currently studied doses, prasugrel inhibits adenosine diphosphate-induced platelet aggregation more rapidly, more consistently, and to a greater extent than do standard and higher doses of clopidogrel in healthy volunteers¹³ and in patients with coronary artery disease,^{14,15} including those undergoing PCI.¹⁶ Phase 2 testing of prasugrel, as compared with clopidogrel, in patients undergoing elective or urgent PCI showed a trend toward fewer ischemic events, with an acceptable safety profile.¹⁷ Thus, we designed the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel—Thrombolysis in Myocardial Infarction (TRITON-TIMI) 38, a phase 3 trial involving patients with acute coronary syndromes with scheduled PCI, comparing a regimen of prasugrel with the standard-dose regimen of clopidogrel approved by the Food and Drug Administration.¹⁸ Although our trial was designed to compare regimens of prasugrel and clopidogrel, it also tests the hypothesis that the use of an agent producing a higher level of inhibition of adenosine diphosphate-induced platelet aggregation and a less-variable response than standard-dose clopidogrel reduces ischemic events.

METHODS

TRITON-TIMI 38 was designed as a collaboration between the TIMI Study Group, the sponsors — Daiichi Sankyo and Eli Lilly — and a steering committee of investigators (see the Appendix).

Quintiles Corporation provided data- and site-management services. All key prespecified and exploratory analyses were performed by the TIMI Study Group, using an independent copy of the complete database. The academic authors wrote all drafts of the manuscript and vouch for the veracity and completeness of its content. The database was locked on September 22, 2007; the analyses reported herein were completed on October 26, 2007.

STUDY POPULATION

We enrolled 13,608 patients with acute coronary syndromes (representative of the entire spectrum of those syndromes) with scheduled PCI. Patients were randomly assigned to the clopidogrel group or the prasugrel group in two strata: 10,074 patients with moderate-to-high-risk unstable angina or non-ST-elevation myocardial infarction and 3534 patients with ST-elevation myocardial infarction. The inclusion criteria for patients with unstable angina or non-ST-elevation myocardial infarction were ischemic symptoms lasting 10 minutes or more and occurring within 72 hours before randomization, a TIMI risk score¹⁹ of 3 or more, and either ST-segment deviation of 1 mm or more or elevated levels of a cardiac biomarker of necrosis. Patients with ST-elevation myocardial infarction could be enrolled within 12 hours after the onset of symptoms if primary PCI was planned or within 14 days after receiving medical treatment for ST-elevation myocardial infarction.¹⁸

Full exclusion criteria have been published previously.¹⁸ Key exclusion criteria included an increased risk of bleeding, anemia, thrombocytopenia, a history of pathologic intracranial findings, or the use of any thienopyridine within 5 days before enrollment.¹⁸ The protocol was approved by the institutional review boards associated with all participating centers, and written informed consent was provided by all patients.

STUDY PROTOCOL

A loading dose of study medication (60 mg of prasugrel or 300 mg of clopidogrel) was administered, in a double-blind manner, anytime between randomization and 1 hour after leaving the cardiac catheterization laboratory. Since the protocol was designed as a trial of patients with acute coronary syndromes who were undergoing PCI, the coronary anatomy had to be known to be suitable for PCI before randomization in all patients with unstable angina or non-ST-elevation myocardial infarction, or in those enrolled after medical treat-

ment for ST-elevation myocardial infarction. If the coronary anatomy was previously known or primary PCI for ST-elevation myocardial infarction was planned, pretreatment with the study drug was permitted for up to 24 hours before PCI. Randomization was to occur before PCI was performed, and the study drug was to be administered as soon as possible after randomization.

The choice of vessels treated, devices used, and adjunctive medication administered to support PCI was left to the discretion of the treating physician. After PCI, patients received maintenance doses of either prasugrel (10 mg) or clopidogrel (75 mg) daily. Use of aspirin was required, and a daily dose of 75 to 162 mg was recommended. Study visits were conducted at hospital discharge, at 30 days, at 90 days, and at 3-month intervals thereafter, for a total of 6 to 15 months.¹⁸

END POINTS

The primary efficacy end point was a composite of the rate of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke during the follow-up period. Key secondary end points at 30 and 90 days were the primary composite end point and a composite of death from cardiovascular causes, nonfatal myocardial infarction, or urgent target-vessel revascularization. Key secondary end points for the entire follow-up period were stent thrombosis and a composite of death from cardiovascular causes, nonfatal myocardial infarction, nonfatal stroke, or rehospitalization due to a cardiac ischemic event. Additional prespecified analyses included an analysis of the rates of the primary end point from randomization to day 3 and a landmark analysis of those data from day 3 to the end of the study. Key safety end points were TIMI major bleeding not related to coronary-artery bypass grafting (CABG), non-CABG-related TIMI life-threatening bleeding, and TIMI major or minor bleeding, as previously defined.¹⁸ Stent thrombosis was defined as definite or probable stent thrombosis according to the Academic Research Consortium.²⁰ All components of the primary, secondary, and key safety end points were adjudicated by members of an independent clinical events committee that was unaware of the group assignments.

STATISTICAL ANALYSIS

Efficacy comparisons were performed on the basis of the time to the first event, according to the intention-to-treat principle. Safety analyses were

carried out on data from patients who received at least one dose of the study drug. The Gehan-Wilcoxon test was used to compare the treatment groups with regard to the primary efficacy end point¹⁸; the log-rank test was used in a prespecified sensitivity analysis for the primary end point and in all analyses of key secondary and safety end points. Because of the substantial overlap between the cohort of patients with unstable angina or non-ST-elevation myocardial infarction and the overall population of patients with acute coronary syndromes, and to preserve the statistical power to detect a difference between the two treatment groups, we used a closed testing procedure. The primary efficacy end point was analyzed in the cohort with unstable angina or non-ST-elevation myocardial infarction first, and only if there was a statistically significant difference between the treatment groups was this end point analyzed in the overall cohort.¹⁸ Rates of the end points are expressed as Kaplan-Meier estimates at 15 months and were compared with the use of hazard ratios and two-sided 95% confidence intervals. An independent data monitoring committee monitored the safety and efficacy of the study drugs. P values of less than 0.05 were considered to indicate statistical significance.

We calculated that a total of 875 primary end points would be required for the study to have a 90% power to detect a 20% reduction in the relative risk of the primary end point among patients with unstable angina or non-ST-elevation myocardial infarction receiving prasugrel, as compared with clopidogrel. It was estimated that 9500 patients with unstable angina or non-ST-elevation myocardial infarction would need to be enrolled to achieve this number of end points.¹⁸ A prespecified assessment conducted when 650 patients had had a primary end point found a slightly lower-than-expected aggregate rate of the end point, which led us to increase the number of patients in the cohort with unstable angina or non-ST-elevation myocardial infarction to approximately 10,100.¹⁸

RESULTS

We randomly assigned 13,608 patients (10,074 with unstable angina or non-ST-elevation myocardial infarction and 3534 with ST-elevation myocardial infarction), from 707 sites in 30 countries, to a treatment group between November 2004 and January 2007. The baseline characteristics were sim-

ilar to those in contemporary studies of patients with acute coronary syndromes who were undergoing PCI and were well matched between the treatment groups (Table 1). The median duration of therapy was 14.5 months. A total of 14 patients (0.1%) were lost to follow-up.

Nearly all patients (99%) had PCI at the time of randomization, 94% received at least one intracoronary stent, and 47% received at least one drug-eluting stent. The study drug was administered before the first coronary guidewire was placed in 25% of patients, after the first coronary guidewire was placed and during the PCI or within 1 hour after PCI in 74%, and more than 1 hour after PCI in 1%.

EFFICACY END POINTS

The rate of the primary efficacy end point was significantly reduced in favor of prasugrel among the patients with unstable angina or non-ST-elevation myocardial infarction (hazard ratio, 0.82; 95% confidence interval [CI], 0.73 to 0.93; $P=0.002$); therefore, as prespecified, the analysis was also performed in the overall cohort of patients with acute coronary syndromes. A significant benefit of prasugrel was also observed in the ST-elevation myocardial infarction cohort alone (hazard ratio, 0.79; 95% CI, 0.65 to 0.97; $P=0.02$), and there was no significant interaction between treatment group and enrollment stratum (unstable angina or non-ST-elevation myocardial infarction vs. ST-elevation myocardial infarction).

In the overall cohort, a total of 781 patients (12.1%) in the clopidogrel group had the primary end point, as compared with 643 patients (9.9%) in the prasugrel group (hazard ratio, 0.81; 95% CI, 0.73 to 0.90; $P<0.001$) (Table 2 and Fig. 1A), supporting the primary hypothesis of superior efficacy. A significant reduction in the primary end point was seen in the prasugrel group by the first prespecified time point, 3 days (5.6% in the clopidogrel group vs. 4.7% in the prasugrel group; hazard ratio, 0.82; 95% CI, 0.71 to 0.96; $P=0.01$) (Fig. 1B), and persisted throughout the follow-up period. From 3 days to the end of the study, the primary end point had occurred in 6.9% of patients receiving clopidogrel and in 5.6% of patients receiving prasugrel (hazard ratio, 0.80; 95% CI, 0.70 to 0.93; $P=0.003$) (Fig. 1C). The difference between the treatment groups with regard to the rate of the primary end point was largely related to a significant reduction in myocardial infarction in the prasugrel group (9.7% in the clopidogrel group vs. 7.4% in the prasugrel group; hazard ratio, 0.76; 95% CI, 0.67 to 0.85; $P<0.001$). The rate of myocardial infarction with subsequent death from cardiovascular causes (including arrhythmia, congestive heart failure, shock, and sudden or unwitnessed death) was also reduced in the prasugrel group (0.7% in the clopidogrel group vs. 0.4% in the prasugrel group; hazard ratio, 0.58; 95% CI, 0.36 to 0.93; $P=0.02$). There was no significant difference between the two treatment groups in the rate of stroke or of death from cardiovascular causes not preceded by recurrent myocardial infarction.

Prasugrel showed superior efficacy in major prespecified subgroups (Fig. 2), without significant interactions between the characteristics of the patients and the treatment group. A benefit with prasugrel with regard to the primary end point was found both with the use of glycoprotein IIb/IIIa-receptor antagonists during the index hospitalization (hazard ratio for prasugrel vs. clopidogrel, 0.79; 95% CI, 0.69 to 0.91; $P<0.001$) or without such use (hazard ratio, 0.84; 95% CI, 0.72 to 0.99; $P=0.03$). The benefit tended to be greater among the 3146 patients with diabetes (17.0% of whom had the primary end point in the clopidogrel group, vs. 12.2% in the prasugrel group; hazard ratio, 0.70; 95% CI, 0.58 to 0.85; $P<0.001$) than among the 10,462 patients without diabetes (10.6% of whom had the primary end point in the clopidogrel group, vs. 9.2% in the prasugrel group; hazard ratio, 0.86; 95% CI, 0.76 to 0.98; $P=0.02$). There was no significant interaction between treatment effect and diabetes status ($P=0.09$) or the timing of the study-drug administration ($P=0.40$).

Similar significant reductions were seen for prasugrel in the overall cohort with regard to the prespecified secondary end point of death from cardiovascular causes, nonfatal myocardial infarction, or urgent target-vessel revascularization at 30 days (hazard ratio, 0.78; 95% CI, 0.69 to 0.89; $P<0.001$) and at 90 days (hazard ratio, 0.79; 95% CI, 0.70 to 0.90; $P<0.001$). A significant reduction in the rate of urgent target-vessel revascularization alone was also found in the prasugrel group by the end of the follow-up period (hazard ratio, 0.66; 95% CI, 0.54 to 0.81; $P<0.001$) (Table 2). A reduction in favor of prasugrel was also seen by the end of the follow-up period for the end point of death from cardiovascular causes, nonfatal myocardial infarction, nonfatal stroke, or rehos-

Similar significant reductions were seen for prasugrel in the overall cohort with regard to the prespecified secondary end point of death from cardiovascular causes, nonfatal myocardial infarction, or urgent target-vessel revascularization at 30 days (hazard ratio, 0.78; 95% CI, 0.69 to 0.89; $P<0.001$) and at 90 days (hazard ratio, 0.79; 95% CI, 0.70 to 0.90; $P<0.001$). A significant reduction in the rate of urgent target-vessel revascularization alone was also found in the prasugrel group by the end of the follow-up period (hazard ratio, 0.66; 95% CI, 0.54 to 0.81; $P<0.001$) (Table 2). A reduction in favor of prasugrel was also seen by the end of the follow-up period for the end point of death from cardiovascular causes, nonfatal myocardial infarction, nonfatal stroke, or rehos-

pitalization for ischemia (hazard ratio, 0.84; 95% CI, 0.76 to 0.92; $P<0.001$) (Table 2). The rate of definite or probable stent thrombosis, as defined by the Academic Research Consortium, was significantly reduced in the prasugrel group as compared with the clopidogrel group, with 68 patients (1.1%) and 142 patients (2.4%), respectively, having at least one occurrence (hazard ratio, 0.48; 95% CI, 0.36 to 0.64; $P<0.001$). The significant reduction in the rate of stent thrombosis was also found among patients receiving prasugrel in combination with bare-metal stents alone (hazard ratio, 0.52; 95% CI, 0.35 to 0.77; $P<0.001$) and among those receiving prasugrel in combination with at least one drug-eluting stent (hazard ratio, 0.43; 95% CI, 0.28 to 0.66; $P<0.001$).

SAFETY END POINTS

Among patients treated with prasugrel, 146 (2.4%) had at least one TIMI major hemorrhage that was not related to CABG, as compared with 111 patients (1.8%) treated with clopidogrel (hazard ratio, 1.32; 95% CI, 1.03 to 1.68; $P=0.03$) (Table 3). This excess of TIMI major bleeding included a higher rate of life-threatening bleeding in the prasugrel group (1.4%, vs. 0.9% in the clopidogrel group; hazard ratio, 1.52; 95% CI, 1.08 to 2.13; $P=0.01$) at the end of the study, as well as from the time of randomization to day 3 (0.4% vs. 0.3%; hazard ratio, 1.38; 95% CI, 0.79 to 2.41; $P=0.26$) and from day 3 to the end of the study (1.0% vs. 0.6%; hazard ratio, 1.60; 95% CI, 1.05 to 2.44; $P=0.03$). Fatal TIMI major bleeding occurred in significantly more patients treated with prasugrel (0.4%) than those treated with clopidogrel (0.1%) ($P=0.002$) (Table 3), and more patients in the prasugrel group had nonfatal life-threatening bleeding (1.1%, vs. 0.9% in the clopidogrel group; hazard ratio, 1.25; 95% CI, 0.87 to 1.81; $P=0.23$). A higher rate of TIMI major bleeding related to instrumentation and a significantly higher rate of spontaneous TIMI major bleeding were seen in the prasugrel group than in the clopidogrel group (Table 3). Intracranial hemorrhage was reported in 19 patients (0.3%) receiving prasugrel and 17 patients (0.3%) receiving clopidogrel ($P=0.74$). The combination of non-CABG-related TIMI major or minor hemorrhage was more frequent among patients receiving prasugrel than among those receiving clopidogrel (hazard ratio, 1.31; 95% CI, 1.11 to 1.56; $P=0.002$) (Table 3).

Few patients underwent CABG; among them,

the rate of TIMI major bleeding was also greater with prasugrel than with clopidogrel (Table 3). More patients treated with prasugrel (2.5%, vs. 1.4% of patients treated with clopidogrel; $P<0.001$) discontinued the study drug owing to adverse events related to hemorrhage.

When the rates of certain efficacy and bleeding end points — death from any cause, nonfatal myocardial infarction, nonfatal stroke, and TIMI major hemorrhage — were included in a prespecified analysis of net clinical benefit, the findings favored prasugrel (13.9% of patients in the clopidogrel group vs. 12.2% in the prasugrel group; hazard ratio, 0.87; 95% CI, 0.79 to 0.95; $P=0.004$). Death from cardiovascular causes (including death related to intracranial hemorrhage or to bleeding related to a cardiovascular procedure) or fatal hemorrhage occurred in 151 patients (2.4%) receiving clopidogrel and in 142 patients (2.2%) receiving prasugrel (hazard ratio, 0.94; 95% CI, 0.75 to 1.18; $P=0.59$).

As a result of the discordance between the efficacy results (lower rates of ischemic end points in the prasugrel group than in the clopidogrel group) and the safety results (higher rates of bleeding end points with prasugrel than with clopidogrel) during the entire follow-up period, we performed a series of post hoc exploratory analyses to identify the subgroups of patients who did not have a favorable net clinical benefit (defined as the rate of death from any cause, nonfatal myocardial infarction, nonfatal stroke, or non-CABG-related nonfatal TIMI major bleeding) from the use of prasugrel or who had net harm. There were three specific groups of interest; patients who had a previous stroke or transient ischemic attack had net harm from prasugrel (hazard ratio, 1.54; 95% CI, 1.02 to 2.32; $P=0.04$), patients 75 years of age or older had no net benefit from prasugrel (hazard ratio, 0.99; 95% CI, 0.81 to 1.21; $P=0.92$), and patients weighing less than 60 kg had no net benefit from prasugrel (hazard ratio, 1.03; 95% CI, 0.69 to 1.53; $P=0.89$). In both treatment groups, patients with at least one of these three risk factors had higher rates of bleeding than those without them (Table 4). Patients with a history of cerebrovascular events had no evidence of a clinical benefit from prasugrel (as compared with clopidogrel), as evaluated by the primary efficacy end point, and had a strong trend toward a greater rate of TIMI major bleeding ($P=0.06$), including intracranial hemor-

Table 1. Baseline Characteristics of the Patients.*

Characteristic	Prasugrel (N=6813)	Clopidogrel (N=6795)
Unstable angina or NSTEMI (%)	74	74
STEMI (%)	26	26
Age		
Median (yr)	61	61
25th percentile, 75th percentile (yr)	53, 69	53, 70
≥75 yr (%)	13	13
Female sex (%)	25	27
BMI†		
Median	28	28
25th percentile, 75th percentile	25, 31	25, 31
White race (%)‡	92	93
Region of enrollment (%)		
North America	32	32
Western Europe	26	26
Eastern Europe	24	25
Middle East, Africa, or Asia-Pacific region	14	14
South America	4	4
Medical history (%)		
Hypertension	64	64
Hypercholesterolemia	56	56
Diabetes mellitus	23	23
Tobacco use	38	38
Previous MI	18	18
Previous CABG	8	7
Creatinine clearance <60 ml/min (%)§	11	12
Index procedure (%)		
PCI	99	99
CABG	1	1
Stent	94	95
Bare-metal stent only	48	47
≥1 Drug-eluting stent	47	47
Multivessel PCI	14	14
Antithrombin use to support PCI (%)		
Heparin	66	65
LMWH	9	8
Bivalirudin	3	3
Other or multiple therapies	22	23
Glycoprotein IIb/IIIa-receptor antagonist use during index hospitalization (%)	54	55
Timing of study-drug administration (%)¶		
Before PCI	26	25
During PCI	73	74
After PCI	1	1

Table 1. (Continued.)

Characteristic	Prasugrel (N=6813)	Clopidogrel (N=6795)
Pharmacotherapy during index hospitalization (%)		
ACE inhibitor or ARB	76	75
Beta-blocker	88	88
Statin	92	92
Calcium-channel blocker	18	17
Aspirin	99	99

* Patients could have had more than one type of medical history, undergone more than one type of index procedure, or received more than one type of pharmacotherapy during index hospitalization. The percentage of female patients and the percentage of patients who received an angiotensin-converting-enzyme (ACE) inhibitor or angiotensin-receptor blocker (ARB) differed significantly between the prasugrel group and the clopidogrel group ($P=0.02$ and $P=0.03$, respectively). NSTEMI denotes non-ST-elevation myocardial infarction (MI), STEMI ST-elevation MI, CABG coronary-artery bypass grafting, PCI percutaneous coronary intervention, and LMWH low-molecular-weight heparin. Beta-blocker is defined as β -adrenergic-receptor antagonist, and statin is defined as hydroxymethylglutaryl-coenzyme A reductase inhibitor.

† The body-mass index (BMI) is the weight in kilograms divided by the square of the height in meters.

‡ Race was self-reported.

§ Creatinine clearance was estimated with the use of the Cockcroft-Gault formula.

¶ Administration of the study drug before PCI occurred before the first coronary guidewire was placed during the index PCI; administration during PCI occurred after the first coronary guidewire was placed or within 1 hour after the patient was taken from the cardiac catheterization laboratory; and administration after PCI occurred more than 1 hour after the patient was taken from the cardiac catheterization laboratory.

rhage in six patients (2.3%) in the prasugrel group, as compared with none in the clopidogrel group ($P=0.02$). As a result, there was a significant interaction between a history of cerebrovascular events and the degree of net clinical benefit of prasugrel as compared with clopidogrel (Table 4), indicating a significant harm from prasugrel among patients with a history of cerebrovascular events (518 patients), as compared with a significant benefit from prasugrel among patients without such a history (13,090 patients). There was also a significant interaction between the presence or absence of any of these three risk factors and the degree of net clinical benefit for prasugrel as compared with clopidogrel ($P=0.006$), though no significant harm was evident. Among patients without any of these three risk factors, there was greater efficacy with prasugrel (hazard ratio, 0.74; 95% CI, 0.66 to 0.84; $P<0.001$), no significant difference in the rate of major bleeding in the prasugrel group and the clopidogrel group (hazard ratio, 1.24; 95% CI, 0.91 to 1.69; $P=0.17$), and a substantially favorable net clinical benefit for the use of prasugrel (Table 4).

The rate of serious adverse events not related to hemorrhage was similar in the prasugrel group and the clopidogrel group (occurring in 22.5% and 22.8% of patients, respectively; $P=0.52$). The study

drug was discontinued owing to adverse events not related to hemorrhage in 4.7% of patients treated with prasugrel and in 5.0% of patients treated with clopidogrel ($P=0.37$). The adverse events reported included severe thrombocytopenia in 17 patients in the prasugrel group (0.3%) and 18 patients in the clopidogrel group (0.3%) ($P=0.86$); neutropenia in 2 patients ($<0.1\%$) and 10 patients (0.2%) ($P=0.02$), respectively; and colonic neoplasms in 13 patients (0.2%) and 4 patients (0.1%) ($P=0.03$). Known gastrointestinal bleeding preceded the diagnosis of colonic neoplasms in nine patients (seven in the prasugrel group and two in the clopidogrel group).

DISCUSSION

The risk of myocardial ischemic events in patients with acute coronary syndromes has been shown to be reduced by means of platelet inhibition with the use of aspirin²¹ and, even more effectively as compared with the use of aspirin alone, dual-antiplatelet therapy with aspirin and ticlopidine or clopidogrel, two inhibitors of the P2Y₁₂ adenosine-diphosphate receptor.^{1-3,5} Our results show that the treatment of patients with acute coronary syndromes, across the full spectrum of such syndromes, with prasugrel (a 60-mg loading dose,

Table 2. Major Efficacy End Points in the Overall Cohort at 15 Months.*

End Point	Prasugrel (N=6813) no. of patients (%)	Clopidogrel (N=6795) no. of patients (%)	Hazard Ratio for Prasugrel (95% CI)	P Value†
Death from cardiovascular causes, nonfatal MI, or nonfatal stroke (primary end point)	643 (9.9)	781 (12.1)	0.81 (0.73–0.90)	<0.001
Death from cardiovascular causes	133 (2.1)	150 (2.4)	0.89 (0.70–1.12)	0.31
Nonfatal MI	475 (7.3)	620 (9.5)	0.76 (0.67–0.85)	<0.001
Nonfatal stroke	61 (1.0)	60 (1.0)	1.02 (0.71–1.45)	0.93
Death from any cause	188 (3.0)	197 (3.2)	0.95 (0.78–1.16)	0.64
Death from cardiovascular causes, nonfatal MI, or urgent target-vessel revascularization	652 (10.0)	798 (12.3)	0.81 (0.73–0.89)	<0.001
Death from any cause, nonfatal MI, or nonfatal stroke	692 (10.7)	822 (12.7)	0.83 (0.75–0.92)	<0.001
Urgent target-vessel revascularization	156 (2.5)	233 (3.7)	0.66 (0.54–0.81)	<0.001
Death from cardiovascular causes, nonfatal MI, nonfatal stroke, or rehospitalization for ischemia	797 (12.3)	938 (14.6)	0.84 (0.76–0.92)	<0.001
Stent thrombosis‡	68 (1.1)	142 (2.4)	0.48 (0.36–0.64)	<0.001

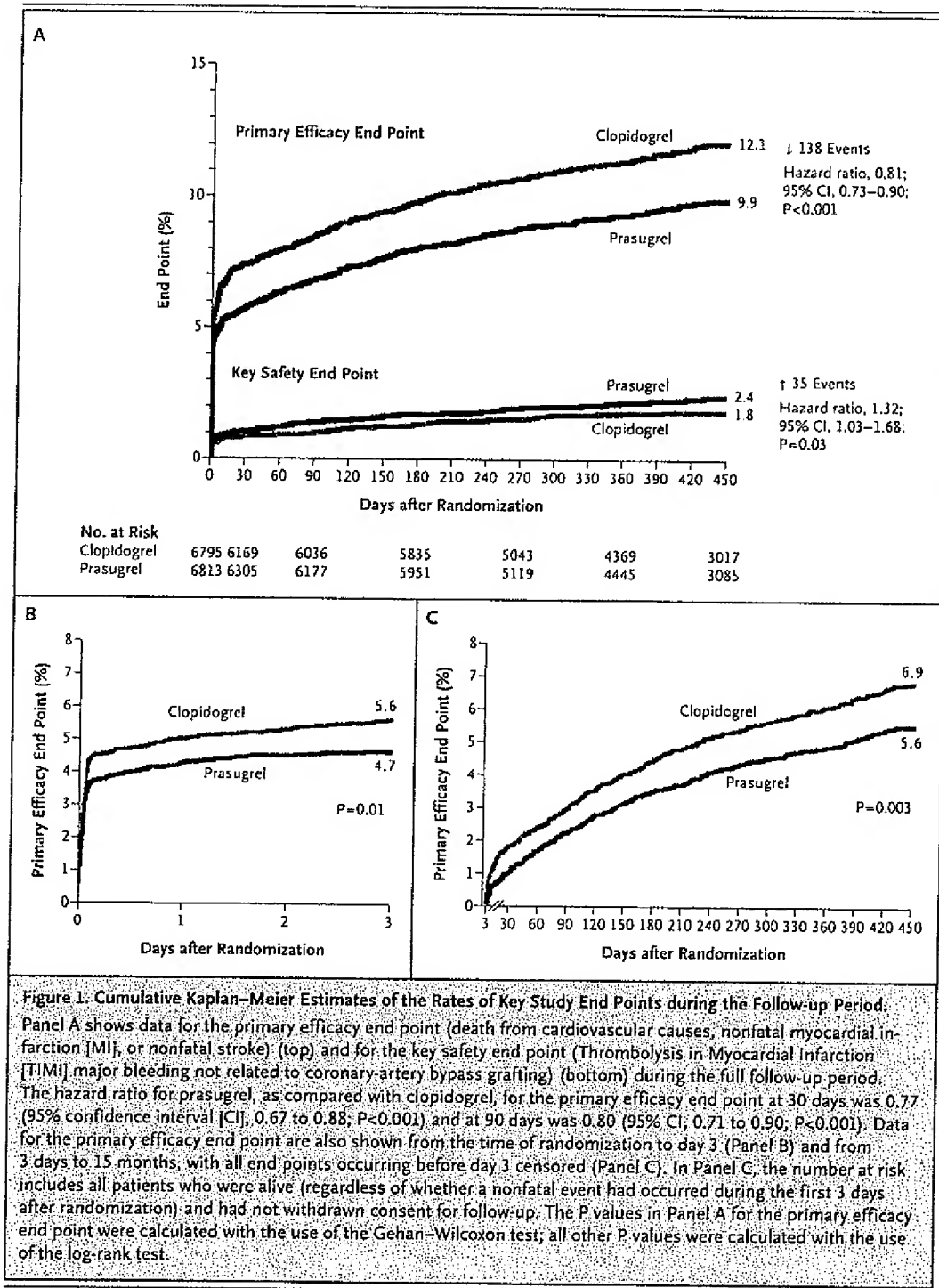
* The percentages are Kaplan–Meier estimates of the rate of the end point at 15 months. Patients could have had more than one type of end point. Death from cardiovascular causes and fatal bleeding (Table 3) are not mutually exclusive, since intracranial hemorrhage and death after cardiovascular procedures that were complicated by fatal bleeding were included in both end points. MI denotes myocardial infarction.

† P values were calculated with the use of the log-rank test. The prespecified analysis for the primary end point used the Gehan–Wilcoxon test, for which the P value was less than 0.001.

‡ Stent thrombosis was defined as definite or probable thrombosis, according to the Academic Research Consortium; the numbers of patients at risk were all patients whose index procedure included at least one intracoronary stent: 6422 patients in each of the two treatment groups.

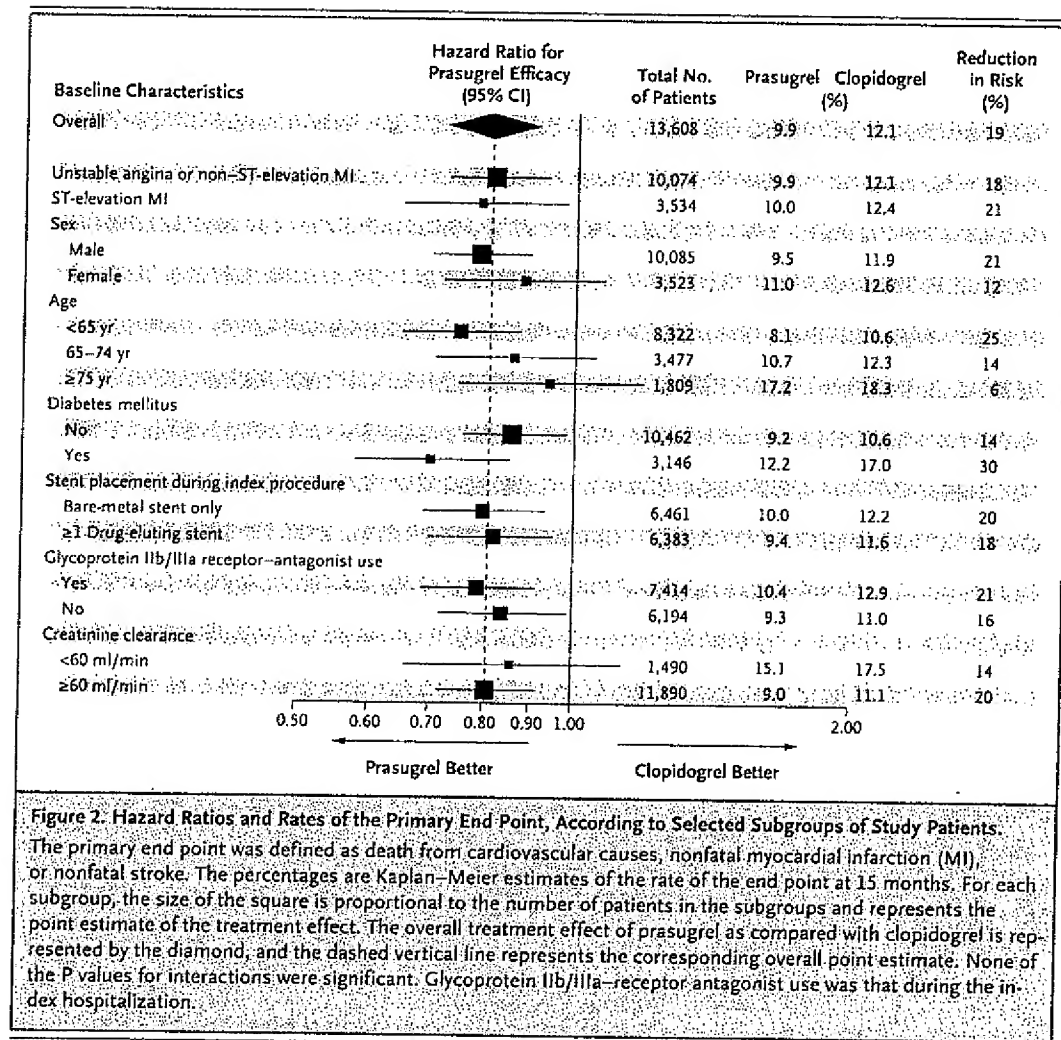
followed by a 10-mg maintenance dose), as compared with clopidogrel at the standard, approved dose, resulted in a significant 2.2% absolute reduction and a 19% relative reduction in the rate of the primary efficacy end point (death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke). The rates of ischemic events were also reduced in the prasugrel group, with a 2.3% absolute reduction and a 24% relative reduction for myocardial infarction, a 1.2% absolute reduction and a 34% relative reduction for urgent target-vessel revascularization, and a 1.3% absolute reduction and a 52% relative reduction for stent thrombosis, a rare but potentially devastating clinical event. Our study was not powered to detect a reduction in the rate of death from cardiovascular causes, and no significant benefit was seen for prasugrel over clopidogrel. However, a 0.3% absolute reduction and a 42% relative reduction were found for recurrent myocardial infarction followed by death from cardiovascular causes.

The reduction in the rate of ischemic events by means of antiplatelet agents, including both oral agents (aspirin and clopidogrel)^{1,21} and intravenous agents (glycoprotein IIb/IIIa-receptor antagonists),^{22–24} has uniformly been accompanied by an increase in bleeding. The Antithrombotic Trialists' Collaboration reported a proportional increase in the odds of major bleeding of 60% with the use of antiplatelet agents (largely aspirin), as compared with placebo.²¹ In the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial, therapy with clopidogrel plus aspirin, as compared with aspirin alone, was associated with a 38% increase in the odds of major bleeding.⁴ The reduction in ischemic events we observed with prasugrel as compared with standard-dose clopidogrel was, as expected, associated with a significant increase in the rate of bleeding. The relative rate of TIMI major hemorrhage was increased by 32% with prasugrel (Table 3). There was an increase in the rate of life-threatening bleeding with prasugrel, including a significant increase in fatal



major hemorrhage. Bleeding episodes, including major or life-threatening hemorrhage, were more frequent in the prasugrel group than in the clopidogrel group, both near the time of PCI and after

PCI. Though few patients underwent CABG, major bleeding occurred at a higher rate among those receiving prasugrel than among those receiving clopidogrel. This finding suggests that, with a



strategy of more potent platelet inhibition, greater attention to the discontinuation of therapy before surgery may be needed.²⁵

Although the results of post hoc subgroup analyses should be considered exploratory, we identified three subgroups of interest that had less clinical efficacy and greater absolute levels of bleeding than the overall cohort, resulting in less net clinical benefit or in clinical harm. These included patients with a history of stroke or transient ischemic attack before enrollment, the elderly (age ≥75 years), and those with a body weight of less than 60 kg, risk factors that have been previously identified as being associated with an increased risk of adverse outcomes from the use of antiplatelet or antithrombotic agents.^{26,27} Patients who had had a cerebrovascular event before en-

rollment in our trial had numerically worse clinical outcomes, as measured in terms of the primary end point, and more frequent bleeding (including intracranial bleeding) than did those without such a history. In previous studies of patients with stroke,²⁸ dual-antiplatelet therapy has been associated with an increased risk of adverse outcomes, particularly intracranial bleeding, as compared with single-antiplatelet therapy. We therefore believe that our findings regarding prasugrel among patients with a history of cerebrovascular events add to the concerns about the risk of intensive inhibition of platelet aggregation in this population. Among the elderly and among patients with a body weight of less than 60 kg in whom neither net benefit nor net harm was observed, it would be expected that increased levels

Table 3. Thrombolysis in Myocardial Infarction (TIMI) Bleeding End Points in the Overall Cohort at 15 Months.*

End Point	Prasugrel (N=6741) no. of patients (%)	Clopidogrel (N=6716) no. of patients (%)	Hazard Ratio for Prasugrel (95% CI)	P Value
Non-CABG-related TIMI major bleeding (key safety end point)	146 (2.4)	111 (1.8)	1.32 (1.03–1.68)	0.03
Related to instrumentation	45 (0.7)	38 (0.6)	1.18 (0.77–1.82)	0.45
Spontaneous	92 (1.6)	61 (1.1)	1.51 (1.09–2.08)	0.01
Related to trauma	9 (0.2)	12 (0.2)	0.75 (0.32–1.78)	0.51
Life-threatening†	85 (1.4)	56 (0.9)	1.52 (1.08–2.13)	0.01
Related to instrumentation	28 (0.5)	18 (0.3)	1.55 (0.86–2.81)	0.14
Spontaneous	50 (0.9)	28 (0.5)	1.78 (1.12–2.83)	0.01
Related to trauma	7 (0.1)	10 (0.2)	0.70 (0.27–1.84)	0.47
Fatal‡	21 (0.4)	5 (0.1)	4.19 (1.58–11.11)	0.002
Nonfatal	64 (1.1)	51 (0.9)	1.25 (0.87–1.81)	0.23
Intracranial	19 (0.3)	17 (0.3)	1.12 (0.58–2.15)	0.74
Major or minor TIMI bleeding	303 (5.0)	231 (3.8)	1.31 (1.11–1.56)	0.002
Bleeding requiring transfusion§	244 (4.0)	182 (3.0)	1.34 (1.11–1.63)	<0.001
CABG-related TIMI major bleeding¶	24 (13.4)	6 (3.2)	4.73 (1.90–11.82)	<0.001

* The data shown are for patients who received at least one dose of the study drug and for end points occurring within 7 days after the study drug was discontinued or occurring within a longer period if the end point was believed by the local investigator to be related to the use of the study drug. Percentages are Kaplan–Meier estimates of the rate of the end point at 15 months. Patients could have had more than one type of end point. CABG denotes coronary-artery bypass grafting.

† The most frequent sites of life-threatening bleeding were gastrointestinal sites, intracranial sites, the puncture site, and retroperitoneal sites.

‡ One patient in the clopidogrel group had a fatal gastrointestinal hemorrhage while receiving the study medication, but hemoglobin testing was not performed and, therefore, the criteria for TIMI major bleeding (including life-threatening and fatal bleeding) could not be applied and the data do not appear in this table.

§ Transfusion was defined as any transfusion of whole blood or packed red cells.

¶ For major bleeding related to CABG, the total number of patients were all patients who had received at least one dose of prasugrel or clopidogrel before undergoing CABG: 179 and 189, respectively. The ratio is the odds ratio, rather than the hazard ratio, and was evaluated with the use of the Cochran–Mantel–Haenszel test.

of the active metabolite of prasugrel may have led to an increased risk of bleeding, owing to altered disposition of the drug or smaller body size. In contrast, a large majority of patients without any of these risk factors had a significant net clinical benefit with the prasugrel regimen studied, as compared with the clopidogrel regimen (hazard ratio, 0.80; 95% CI, 0.71 to 0.89; $P<0.001$). Additional work to define populations with an increased risk of bleeding, in association with oral regimens yielding high degrees of inhibition of platelet aggregation, is likely to be helpful in guiding therapy.

In addition to the results of our key prespecified safety analyses, we noted a higher rate of adverse events related to colonic cancer in the prasugrel group than in the clopidogrel group. Though

we cannot fully rule out either a possible causative effect or the play of chance, this imbalance may have resulted from the more potent antiplatelet effect of prasugrel bringing more events to medical attention, a phenomenon seen with other anticoagulant agents, including warfarin.^{29,30}

Treatment with prasugrel at the dosage used in our trial has been shown to generate higher and more consistent levels of active metabolite than treatment with approved doses of clopidogrel.¹³ This results in higher levels of mean inhibition of platelet aggregation, lower interpatient variability in such inhibition, and fewer patients considered to have poor responsiveness or hyporesponsiveness when platelet function is assessed in the laboratory.¹³ Considerable research has focused on the presence and clinical meaning of hyporesponsive-

Table 4. The Balance of Efficacy and Safety in Selected Subgroups.*

End Point	Prasugrel no. of patients/total no. (%)	Clopidogrel no. of patients/total no. (%)	Hazard Ratio for Prasugrel (95% CI)	P Value	P Value for Interaction†
History of stroke or TIA					
Death from cardiovascular causes, nonfatal MI, or nonfatal stroke (primary efficacy end point)	47/262 (19.1)	35/256 (14.4)	1.37 (0.89–2.13)	0.15	
Non-CABG-related TIMI major bleeding	14/257 (5.0)	6/252 (2.9)	2.46 (0.94–6.42)	0.06	
Death from any cause, nonfatal MI, nonfatal stroke, or non-CABG-related nonfatal TIMI major bleeding	57/262 (23.0)	39/256 (16.0)	1.54 (1.02–2.32)	0.04	
No history of stroke or TIA					
Death from cardiovascular causes, nonfatal MI, or nonfatal stroke (primary efficacy end point)	596/6551 (9.5)	746/6539 (12.0)	0.79 (0.71–0.88)	<0.001	0.02
Non-CABG-related TIMI major bleeding	132/6484 (2.3)	105/6464 (1.8)	1.26 (0.97–1.62)	0.08	0.22
Death from any cause, nonfatal MI, nonfatal stroke, or non-CABG-related nonfatal TIMI major bleeding	727/6551 (11.8)	854/6539 (13.8)	0.84 (0.76–0.93)	<0.001	0.006
Age ≥75 yr, body weight <60 kg, or history of stroke or TIA					
Death from cardiovascular causes, nonfatal MI, or nonfatal stroke (primary efficacy end point)	198/1320 (16.1)	199/1347 (16.0)	1.02 (0.84–1.24)	0.83	
Non-CABG-related TIMI major bleeding	52/1305 (4.3)	38/1328 (3.3)	1.42 (0.93–2.15)	0.10	
Death from any cause, nonfatal MI, nonfatal stroke, or non-CABG-related nonfatal TIMI major bleeding	249/1320 (20.2)	239/1347 (19.0)	1.07 (0.90–1.28)	0.43	
Age <75 yr, body weight ≥60 kg, and no history of stroke or TIA					
Death from cardiovascular causes, nonfatal MI, or nonfatal stroke (primary efficacy end point)	433/5421 (8.3)	569/5383 (11.0)	0.74 (0.66–0.84)	<0.001	0.008
Non-CABG-related TIMI major bleeding	91/5390 (2.0)	73/5337 (1.5)	1.24 (0.91–1.69)	0.17	0.64
Death from any cause, nonfatal MI, nonfatal stroke, or non-CABG-related nonfatal TIMI major bleeding	522/5421 (10.2)	641/5383 (12.5)	0.80 (0.71–0.89)	<0.001	0.006

* The rates of Thrombolysis in Myocardial Infarction (TIMI) major bleeding not related to coronary-artery bypass grafting (CABG) were calculated as Kaplan–Meier estimates for patients who received at least one dose of the study drug and for end points occurring within 7 days after the study drug was discontinued or occurring within a longer period if the end point was believed by the local investigator to be related to the use of the study drug. The rates of the other end points were calculated as Kaplan–Meier estimates in the intention-to-treat cohort. TIA denotes transient ischemic attack, and MI myocardial infarction.

† P values for interaction were those for the interaction between the status of the risk factor and the hazard ratio for the end point.

ness to clopidogrel in patients with coronary artery disease who have undergone PCI.^{6–11} The data from our trial, which was adequately powered to evaluate clinical events, show that, as compared with standard-dose clopidogrel therapy, a regimen that improves the inhibition of platelet aggregation is associated with fewer ischemic events. This improvement in the rate of ischemic events as a

result of greater platelet inhibition was not assured, given the absence of increased efficacy with higher doses of aspirin³¹ and the higher rates of ischemic events seen with the addition of oral glycoprotein IIb/IIIa-receptor antagonists (potent inhibitors of platelet aggregation) to aspirin.³²

As a result of the intention to have all patients undergo PCI, our trial was largely a comparison of

prasugrel therapy and clopidogrel therapy among patients treated with a thienopyridine at the time of the identification of coronary anatomy appropriate for PCI, rather than a comparison of routine pretreatment with either agent before diagnostic cardiac catheterization. A strategy of clopidogrel loading when coronary anatomy is known is now used by many cardiologists because of concern about surgical bleeding if a patient receives clopidogrel and then (because of a finding on coronary angiography) goes on to undergo CABG.²⁵ Pharmacodynamic data have shown that the degree of inhibition of platelet aggregation achieved with prasugrel within 30 minutes after treatment is similar to the peak effect of clopidogrel 6 hours after administration, suggesting that prolonged pretreatment may not be necessary for prasugrel to achieve its therapeutic effect.¹³ The more rapid onset of an antiplatelet effect with prasugrel than with clopidogrel may have played an important role in the efficacy benefit, an assertion supported by the reduction in the rate of early myocardial infarction (before day 3) (Fig. 1B), despite the lack of pretreatment. However, when considering only end points occurring after day 3 (Fig. 1C), the time at which the use of each drug should have resulted in the steady-state inhibition of platelet aggregation, the significant reduction in the rate of ischemic end points persisted, suggesting a continued benefit of greater inhibition of platelet aggregation during maintenance therapy.

Partly because of data reporting an improved inhibition of platelet aggregation,^{33,34} many clinicians have adopted the use of a higher-than-standard loading dose of clopidogrel in patients with PCI, a practice endorsed by guideline committees.^{35,36} The clinical-efficacy data supporting the use of such higher-dose clopidogrel have been from small studies and have been inconsistent.^{37,38} The use of prasugrel (60 mg) has been shown to result in a greater inhibition of platelet aggregation than the use of clopidogrel (600 mg) in patients with chronic coronary artery disease.¹⁵ The Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation (PRINCIPLE)-TIMI 44 trial¹⁶ showed a markedly superior inhibition of platelet aggregation, with regard to multiple measures of platelet function, in patients who had undergone elective PCI and who had received the regimen of prasugrel used in our study as compared with a higher-than-standard dose regimen of clopidogrel (a 600-mg load-

ing dose and a 150-mg maintenance dose) — though the PRINCIPLE-TIMI 44 trial was not powered to study clinical end points.

In our study of a selected population with moderate-to-high-risk acute coronary syndromes, on average, for every 1000 patients treated with prasugrel as compared with clopidogrel at the doses studied, 23 myocardial infarctions were prevented, with an excess of six non-CABG-related TIMI major hemorrhages. The estimated number of patients needed to be treated with prasugrel at the dosage studied, as compared with standard-dose clopidogrel, to prevent one primary efficacy end point during a 15-month period was 46. The number of patients who would have to be treated to result in an excess non-CABG-related TIMI major hemorrhage was 167.

Our data support the hypothesis that the greater inhibition of adenosine diphosphate-induced platelet aggregation by means of the tested regimen of prasugrel, a potent oral P2Y₁₂ inhibitor, is more effective at preventing ischemic events than is the inhibition conferred by a standard regimen of clopidogrel. However, this beneficial effect is accompanied by an increase in the rate of major bleeding. When considering the choice of antiplatelet regimens for the treatment of patients with acute coronary syndromes who are undergoing PCI, clinicians need to weigh the benefits and risks of intensive inhibition of platelet aggregation.

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APPENDIX

The members of the Operations and Steering Committees of the TRITON-TIMI 38 were as follows (with principal investigators at participating centers listed separately, in the Supplementary Appendix): TIMI Study Group, Brigham and Women's Hospital, Boston: E. Braunwald (study chair), E.M. Antman (principal investigator), S.D. Wiviott (investigator), C.M. Gibson (investigator), C.H. McCabe (director), S.A. Murphy (lead biostatistician), J. Buros (biostatistician), S. McHale (project manager); Sponsors: Eli Lilly — J. Riesmeyer, J.A. Ware, G. Weerakkody, W. Macias, E. Moscarelli, J. Croanings; Daiichi Sankyo — J. Warmke, F. Plat, T. Bocanegra, J. Hanyok, C. Hsu; Data Coordinating Center (Quintiles): K. Long, D. White, S. Boyle; Steering Committee: all members of the TIMI Study Group and sponsor staff listed above; France — G. Montalescot (coprincipal investigator), P.G. Steg; Norway — L. Aaberge; Denmark — H.R. Anderson; Italy — D. Ardissino, S. De Servi; Australia — P. Aylward; Chile — R. Corbalan; South Africa — A. Dalby; Slovak Republic — V. Fridrich; United States — M. Furman, D. Kereiakes, N. Kleiman, J. Popma; Canada — S. Goodman; Israel — S. Gottlieb; Argentina — E. Gurfinkel; Austria — K. Huber; Hungary — M. Keltai; Spain — J. Lopez-Sendon; Switzerland — T. Luscher; Germany — F.-J. Neumann, A. Schömig; Brazil — J. Nicolau; Poland — W. Ruzyllo; Sweden — F. Schersten; Portugal — R. Seabra-Gomes; Iceland — A. Sigurdsson; Finland — M. Syvanne; Belgium — F. Van de Werf; the Netherlands — F. Verheugt; New Zealand — H. White; Czech Republic — P. Widimsky; United Kingdom — R. Wilcox; Data Monitoring Committee: D.O. Williams (chair); D. DeMets (statistician); C. Bode, S. King, U. Sigwart; Clinical Events Committee: B. Scirica (administrative chair), E. Awtrey, C. Berger, S. Bernard, A. Desai, E. Gelfand, C. Ho, F. Jaffer, S. Kathiresan, D. Leeman, M. Link, W. Maisel, F. Ruberg, U. Tedrow, J. Vita, P. Zimerbaum.

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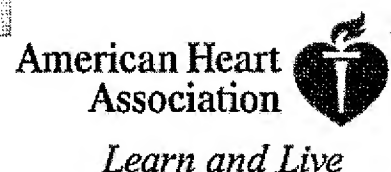
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Core 6. Catheter-based and Surgical Interventions

Session Title: Pharmacology for Acute Coronary Syndromes and Percutaneous Coronary Intervention

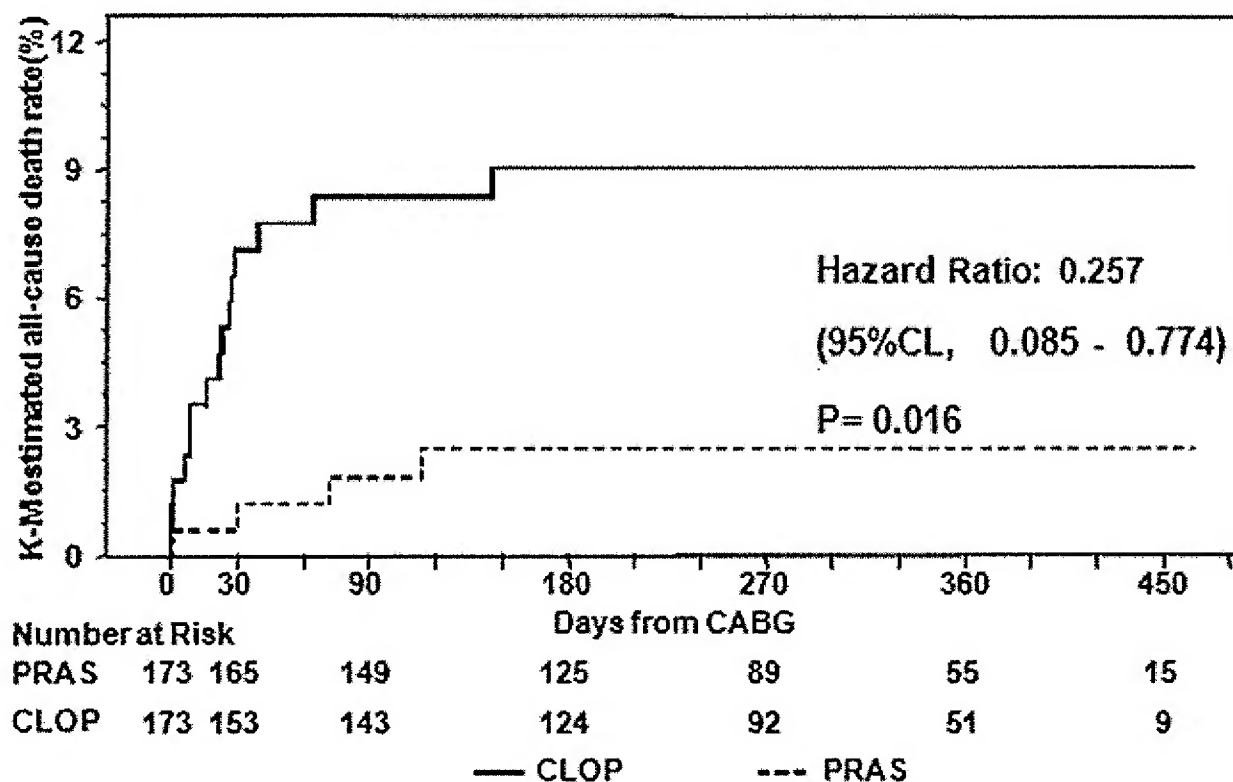
Abstract 10881: Mortality Benefit with Prasugrel in TRITON – TIMI 38 Coronary Artery Bypass Grafting (CABG) Cohort: Risk Adjusted Retrospective Data Analysis

Peter K Smith; George J Despotis; Lawrence T Goodnough; Jerrold H Levy; Robert S Poston; Mary A Short; Govinda J Weerakkody; LeRoy A LeNarz

Duke Univ, Durham, NC; Washington Univ Sch of Medicine, St. Louis, MO; Stanford Univ, Stanford, CA; Emory Univ Hosp, Atlanta, GA; Boston Univ Sch of Medicine, Boston, MA; Lilly USA, LLC, Indianapolis, IN

A cohort of 422 patients undergoing isolated CABG in TRITON-TIMI 38 (PRASugrel n=208; CLOPidogrel n=214) was analyzed to characterize the outcomes of subjects related to timing of study drug withdrawal prior to CABG. Patients who never received study drug (N=56) or who received open label thienopyridine (N=20) treatment prior to CABG were excluded. A significantly lower mortality was observed in the PRAS cohort, see Figure 1. Overall all-cause mortality was 2.31% in the PRAS cohort compared to 8.67% in the CLOP cohort, with a hazard ratio of 0.26 (Log-rank p=0.016). Mortality was similar (3/16 PRAS, 3/14 CLOP) when CABG was performed before study drug was discontinued, but lower when study drug was discontinued for 1 or more days (1/156 PRAS vs 12/158 CLOP). There were no deaths (0/72) in PRAS patients having CABG with 1 to 5 days of drug withdrawal and 6 deaths in CLOP patients having CABG

(6/85). The mortality risk at 30-days adjusted for possible imbalances at CABG baseline when analyzed by logistic regression per EURO scoring ($p=0.024$) remained statistically significant. There was significantly higher mean 12 hr chest tube loss (655 ± 580 ml vs. 503 ± 378 ml; Kruskal-Wallis $p=0.050$) and platelet transfusion (17.96% vs. 9.82%; Pearson's chi-squares $p=.033$) with PRAS. However, there were no clinically important differences in packed red cell transfusion units (mean 2.08 ± 3.00 PRAS vs. 1.71 ± 2.23 CLOP; Kruskal-Wallis $p=0.442$) or total donor exposure units (4.43 ± 7.58 PRAS vs. 3.00 ± 4.54 CLOP; Kruskal-Wallis $p=0.463$). Despite this increase in observed bleeding, PRAS was associated with a lower rate of death in patients undergoing CABG.



Author Disclosures: **P.K. Smith**, Eli Lilly and Company, Modest, Consultant/Advisory Board; Cubist, Modest, Consultant/Advisory Board; CSL Behring, Modest, Consultant/Advisory Board; **G.J. Despotis**, Eli Lilly and Company, Modest, Consultant/Advisory Board; **L.T. Goodnough**, Amgen, Modest, Consultant/Advisory Board; Luitpold, Modest, Consultant/Advisory Board; Amag, Modest, Consultant/Advisory Board; Eli Lilly and Company, Significant, Consultant/Advisory Board; **J.H. Levy**, Eli Lilly and Company, Modest, Research Grant; Eli Lilly and Company, Modest, Honoraria; **R.S. Poston**, None; **M.A. Short**, Eli Lilly and Company, Significant, Employment; Eli Lilly and Company, Significant, Ownership Interest; **G.J. Weerakkody**, Eli Lilly and Company, Significant, Employment; Eli Lilly and Company, Significant, Ownership Interest; **L.A. LeNarz**, Eli Lilly and Company, Significant, Employment; Eli Lilly and Company, Significant, Ownership Interest.

Key Words: Antiplatelet drugs • Acute coronary syndromes

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Exhibit 28

ORIGINAL ARTICLE

Clopidogrel and Aspirin versus Aspirin Alone for the Prevention of Atherothrombotic Events

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*The Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) committees, national coordinators, and investigators are listed in the Appendix. This article was published at www.nejm.org on March 12, 2006.

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ABSTRACT

BACKGROUND

Dual antiplatelet therapy with clopidogrel plus low-dose aspirin has not been studied in a broad population of patients at high risk for atherothrombotic events.

METHODS

We randomly assigned 15,603 patients with either clinically evident cardiovascular disease or multiple risk factors to receive clopidogrel (75 mg per day) plus low-dose aspirin (75 to 162 mg per day) or placebo plus low-dose aspirin and followed them for a median of 28 months. The primary efficacy end point was a composite of myocardial infarction, stroke, or death from cardiovascular causes.

RESULTS

The rate of the primary efficacy end point was 6.8 percent with clopidogrel plus aspirin and 7.3 percent with placebo plus aspirin (relative risk, 0.93; 95 percent confidence interval, 0.83 to 1.05; $P=0.22$). The respective rate of the principal secondary efficacy end point, which included hospitalizations for ischemic events, was 16.7 percent and 17.9 percent (relative risk, 0.92; 95 percent confidence interval, 0.86 to 0.995; $P=0.04$), and the rate of severe bleeding was 1.7 percent and 1.3 percent (relative risk, 1.25; 95 percent confidence interval, 0.97 to 1.61 percent; $P=0.09$). The rate of the primary end point among patients with multiple risk factors was 6.6 percent with clopidogrel and 5.5 percent with placebo (relative risk, 1.2; 95 percent confidence interval, 0.91 to 1.59; $P=0.20$) and the rate of death from cardiovascular causes also was higher with clopidogrel (3.9 percent vs. 2.2 percent, $P=0.01$). In the subgroup with clinically evident atherothrombosis, the rate was 6.9 percent with clopidogrel and 7.9 percent with placebo (relative risk, 0.88; 95 percent confidence interval, 0.77 to 0.998; $P=0.046$).

CONCLUSIONS

In this trial, there was a suggestion of benefit with clopidogrel treatment in patients with symptomatic atherothrombosis and a suggestion of harm in patients with multiple risk factors. Overall, clopidogrel plus aspirin was not significantly more effective than aspirin alone in reducing the rate of myocardial infarction, stroke, or death from cardiovascular causes. (ClinicalTrials.gov number, NCT00050817)

ATHEROSCLEROTIC VASCULAR DISEASE has a propensity to engender arterial thrombosis, a sequence that has been characterized as an "atherothrombotic" process.^{1,2} Collectively, atherothrombotic disorders of the coronary, cerebrovascular, and peripheral arterial circulation are the leading cause of death and disability in the world.³ Their prevalence is increasing; they are significantly undertreated, and better means of prevention are needed.⁴

Platelets have been shown to play a central role in the pathogenesis of atherothrombosis.^{1,2} Low-dose aspirin has been shown to reduce ischemic outcomes in patients above a certain risk threshold.⁵ However, aspirin alone in many instances is not sufficient to prevent ischemic events in patients at high risk. Furthermore, aspirin inhibits only the cyclooxygenase pathway, leaving the adenosine diphosphate P2Y₁₂ receptor unaffected. Dual antiplatelet therapy with clopidogrel (Plavix, Sanofi-Aventis), a P2Y₁₂-receptor antagonist, plus aspirin has been shown to reduce ischemic events in patients with unstable angina, myocardial infarction without ST-segment elevation, or myocardial infarction with ST-segment elevation, as well as those undergoing angioplasty and stenting.⁶⁻⁹

Accordingly, we tested the hypothesis that long-term treatment with a combination of clopidogrel plus aspirin may provide greater protection against cardiovascular events than aspirin alone in a broad population of patients at high risk.

METHODS

TRIAL DESIGN

The Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial was a prospective, multicenter, randomized, double-blind, placebo-controlled study of the efficacy and safety of clopidogrel plus aspirin as compared with aspirin alone in patients at high risk for a cardiovascular event. The details of the trial design have been published previously.¹⁰ The trial was approved by the institutional ethics committee of each participating institution as well as the appropriate national ethics committees.

The trial was designed by Dr. Topol, who was responsible for obtaining funding and executing the trial, and it was planned and conducted by the executive committee, with extensive review of the data for its interpretation. The trial was managed

by the Cleveland Clinic Cardiovascular Coordinating Center and by the national coordinators in each country in which patients were enrolled. Data collection and entry were performed by the sponsor and cosponsor. The locked, cleaned database was transferred to the Cleveland Clinic Cardiovascular Coordinating Center, where data analysis was performed. Dr. Bhatt prepared the first draft of the manuscript, and the executive committee helped to revise it. Dr. Topol had full access to an independent database for any query regarding the analyses and assumes responsibility for the integrity of the data.

Funding for the CHARISMA trial was provided by Sanofi-Aventis and Bristol-Myers Squibb. The sponsor and cosponsor had advisory input in the design of the study, had nonvoting input in the executive committee, and were responsible for auditing at individual study sites. The executive committee bears complete responsibility for the analysis of the results, the veracity and completeness of the reporting, and the writing of the manuscript; the sponsors did have the opportunity to review the manuscript.

PATIENTS

Patients were eligible to enroll in the trial if they were 45 years of age or older and had one of the following conditions: multiple atherothrombotic risk factors, documented coronary disease, documented cerebrovascular disease, or documented symptomatic peripheral arterial disease. The inclusion criteria for those with multiple risk factors and for those with established vascular disease are shown in Table 1.

Patients were excluded from the trial if they were taking oral antithrombotic medications or nonsteroidal antiinflammatory drugs on a long-term basis (although cyclooxygenase-2 inhibitors were permitted). Patients were also excluded if, in the judgment of the investigator, they had established indications for clopidogrel therapy (such as a recent acute coronary syndrome). Patients who were scheduled to undergo a revascularization were not allowed to enroll until the procedure had been completed; such patients were excluded if they were considered to require clopidogrel after revascularization.

TRIAL PROCEDURES

After providing written informed consent, patients were randomly assigned either to clopidogrel (75 mg per day) plus low-dose aspirin (75 to

Table 1. Inclusion Criteria for Patients with Multiple Atherothrombotic Risk Factors and for Those with Established Cardiovascular Disease.

Patients and Criteria	Clopidogrel plus Aspirin	Placebo plus Aspirin
	<i>no. of patients (%)</i>	
Patients with multiple atherothrombotic risk factors*	1659	1625
Major risk factors	1535 (92.5)	1490 (91.7)
Type 1 or 2 diabetes (with drug therapy)	1360 (82.0)	1295 (79.7)
Diabetic nephropathy	716 (43.2)	687 (42.3)
Ankle-brachial index <0.9	94 (5.7)	92 (5.7)
Asymptomatic carotid stenosis $\geq 70\%$ of luminal diameter	123 (7.4)	132 (8.1)
≥ 1 Carotid plaque, as evidenced by intima-media thickness	198 (11.9)	213 (13.1)
Minor risk factors	1474 (88.8)	1454 (89.5)
Systolic blood pressure ≥ 150 mm Hg, despite therapy for at least 3 mo	809 (48.8)	744 (45.8)
Primary hypercholesterolemia	993 (59.9)	1030 (63.4)
Current smoking >15 cigarettes/day	284 (17.1)	271 (16.7)
Male sex and age ≥ 65 yr or female sex and age ≥ 70 yr	841 (50.7)	853 (52.5)
Patients with established cardiovascular disease†	6062	6091
Documented coronary disease	2892 (47.7)	2943 (48.3)
Angina with documented multivessel coronary disease	888 (14.6)	885 (14.5)
History of multivessel percutaneous coronary intervention	398 (6.6)	434 (7.1)
History of multivessel coronary-artery bypass grafting	736 (12.1)	733 (12.0)
Myocardial infarction	1903 (31.4)	1943 (31.9)
Documented cerebrovascular disease	2157 (35.6)	2163 (35.5)
Transient ischemic attack during previous 5 yr	617 (10.2)	616 (10.1)
Ischemic stroke during previous 5 yr	1634 (27.0)	1611 (26.4)
Documented symptomatic peripheral arterial disease	1418 (23.4)	1420 (23.3)
Current intermittent claudication and ankle-brachial index ≤ 0.85	885 (14.6)	892 (14.6)
History of intermittent claudication and previous intervention (e.g., amputation, peripheral bypass, or angioplasty)	835 (13.8)	801 (13.2)

* Data on the other 166 patients enrolled but not categorized were not adequately differentiated on the basis of medical records. To meet the criterion for enrollment on the basis of multiple risk factors, patients were required to have two major or three minor or one major and two minor atherothrombotic risk factors.

† To meet the criterion for enrollment on the basis of established cardiovascular disease, patients were required to have one of the listed conditions.

162 mg per day) or to placebo plus low-dose aspirin. Study-drug assignment was performed centrally by an interactive voice-response system on the basis of a preestablished randomization scheme, stratified according to site. All patients also received standard therapy as appropriate (e.g., statins or beta-blockers) at the discretion of the investigator and other responsible clinicians. The use of appropriate background therapy was emphasized to the investigators, who were provided with international guidelines.

Follow-up evaluations were performed at one

month, three months, and six months and every six months thereafter until the end of the trial. At these visits, patients' compliance was assessed, standard medication was adjusted as appropriate, and all interventions, outcome events, and adverse events were recorded. According to the power calculations described below and the event-driven design of the trial, all patients were followed until a common study end date based on the pre-specified target of 1040 primary efficacy end points was reached.

END POINTS

All primary trial end points were adjudicated by the clinical events committee, whose members were unaware of patients' treatment assignments. The primary efficacy end point was the first occurrence of myocardial infarction, stroke (of any cause), or death from cardiovascular causes (including hemorrhage). The principal secondary efficacy end point was the first occurrence of myocardial infarction, stroke, death from cardiovascular causes, or hospitalization for unstable angina, a transient ischemic attack, or a revascularization procedure (coronary, cerebral, or peripheral). Other efficacy end points included death from any cause and death from cardiovascular causes as well as myocardial infarction, ischemic stroke, any stroke, and hospitalization for unstable angina, transient ischemic attack, or revascularization, considered separately.

The primary safety end point was severe bleeding, according to the Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries (GUSTO) definition, which includes fatal bleeding and intracranial hemorrhage, or bleeding that caused hemodynamic compromise requiring blood or fluid replacement, inotropic support, or surgical intervention.¹² Moderate bleeding according to the GUSTO criteria (bleeding that led to transfusion but did not meet the criteria for severe bleeding) was also examined, as were fatal bleeding and primary intracranial hemorrhage.

Analyses of the primary end point were also performed in several prospectively defined subgroups. The subgroups included symptomatic patients (defined as patients enrolled on the basis of established cardiovascular disease) as compared with asymptomatic patients (those enrolled on the basis of multiple atherothrombotic risk factors), as well as patients with and those without a history of diabetes, hypertension, hypercholesterolemia, peripheral arterial disease, prior cardiac or vascular surgery, prior myocardial infarction, prior stroke, prior transient ischemic attack, or prior use of other antiplatelet agents, angiotensin-converting-enzyme (ACE) inhibitors (overall and ramipril vs. other ACE inhibitors), statins (overall and atorvastatin, simvastatin, and pravastatin), beta-blockers, calcium antagonists, antidiabetic agents, angiotensin II-receptor blockers, cyclooxygenase-2 inhibitors, and anticoagulants.

STATISTICAL ANALYSIS

We estimated that 15,200 patients (7600 per group) and 1040 primary events would be necessary to detect a 20 percent relative risk reduction in the primary efficacy end point, with 90 percent power at the two-sided 0.05 significance level in this event-driven trial, assuming an annual event rate of 3.1 percent in the control group and 18 to 42 months of follow-up. The primary efficacy outcome was monitored with use of a Peto-Haybittle type of stopping rule based on the P value of the log-rank test. Two preplanned interim analyses were conducted by a statistician associated with the independent data and safety monitoring board. A two-sided type I error of 0.001 was used at each analysis. A type I error of 0.049 was preserved for the final analysis.

Data were analyzed on an intention-to-treat basis, with the inclusion of all patients according to their randomly assigned treatment group and the inclusion of outcomes occurring from randomization to a common study end date (August 29, 2005). The time to the first occurrence of any event in the composite cluster was used for analysis. Data on patients who did not reach the primary end point by the study end date were censored on the date of the patients' last assessment visit. Death from noncardiovascular causes was treated as a competing event, and follow-up was censored on the date of death.

The primary efficacy of clopidogrel as compared with placebo was assessed with the use of a two-sided log-rank test. The treatment effect as measured by the hazard ratio (the relative risk) and its associated 95 percent confidence interval was estimated with the use of the Cox proportional-hazards model. Cumulative incidence event curves were also calculated. Statistical comparisons of the primary safety-event rates in the two treatment groups were performed with Pearson's chi-square test. No adjustments for multiple comparisons were made. All analyses were performed with SAS software (version 8.0, SAS Institute).

RESULTS

CHARACTERISTICS OF THE PATIENTS

A total of 15,603 patients from 32 countries and 768 sites were enrolled between October 1, 2002, and November 14, 2003, in the CHARISMA trial. Of these patients, 7802 were assigned to receive

Table 2. Baseline Characteristics.

Characteristic	Clopidogrel plus Aspirin (N=7802)	Placebo plus Aspirin (N=7801)
Demographic characteristics		
Age — yr		
Median	64.0	64.0
Range	39.0–95.0	45.0–93.0
Female sex — no. (%)	2316 (29.7)	2328 (29.8)
Race or ethnic group — no. (%)*		
White	6272 (80.4)	6230 (79.9)
Hispanic	776 (9.9)	837 (10.7)
Asian	387 (5.0)	388 (5.0)
Black	252 (3.2)	234 (3.0)
Other	115 (1.5)	112 (1.4)
Inclusion subgroup		
Documented vascular disease — no. (%)	6062 (77.7)	6091 (78.1)
Multiple risk factors — no. (%)	1659 (21.3)	1625 (20.8)
Neither subgroup — no. (%)	81 (1.0)	85 (1.1)
Selected clinical characteristics		
Smoking status — no. (%)		
Current	1571 (20.1)	1584 (20.3)
Former	3811 (48.9)	3802 (48.7)
Hypertension — no. (%)	5719 (73.3)	5764 (73.9)
Hypercholesterolemia — no. (%)	5748 (73.7)	5787 (74.2)
Congestive heart failure — no. (%)	469 (6.0)	457 (5.9)
Prior myocardial infarction — no. (%)	2672 (34.2)	2725 (34.9)
Atrial fibrillation — no. (%)	298 (3.8)	285 (3.7)
Prior stroke — no. (%)	1942 (24.9)	1895 (24.3)
Prior transient ischemic attack — no. (%)	938 (12.0)	926 (11.9)
Diabetes — no. (%)	3304 (42.3)	3252 (41.7)
Peripheral arterial disease — no. (%)	1760 (22.6)	1771 (22.7)
Prior percutaneous coronary intervention — no. (%)	1750 (22.4)	1804 (23.1)
Prior coronary-artery bypass grafting — no. (%)	1525 (19.5)	1554 (19.9)
Prior carotid endarterectomy	420 (5.4)	405 (5.2)
Prior peripheral angioplasty or bypass — no. (%)	879 (11.3)	858 (11.0)
Diabetic nephropathy — no. (%)	1006 (12.9)	1003 (12.9)

* Race or ethnic group was self-reported.

total of 4.8 percent of the patients in the clopidogrel group and 4.9 percent of those in the placebo group discontinued treatment because of an adverse event ($P=0.67$).

The baseline characteristics of the patients in the trial have been described previously,¹² and selected features are listed in Table 2. The median age was 64 years; 29.8 percent of the patients were women. More than three quarters of the participants had established cardiovascular disease, as defined by the enrollment criteria, and most of the remaining patients had multiple atherothrombotic risk factors. On retrospective review of the enrollment information, 166 patients did not fall into either of these categories but were still considered in the broad population analysis.

Medications taken by the patients are shown in Table 3; these figures indicate the maximal frequency of use of each agent at any time during the trial (with use assessed at baseline and at every follow-up visit). Almost all the patients (aside from those who died or dropped out) took aspirin and the study drug, and 10.2 percent also took open-label clopidogrel. Three quarters took a statin, and more than half took a beta-blocker. Nearly two thirds took an ACE inhibitor, and a quarter took angiotensin II-receptor blocking agents.

EFFICACY END POINTS

Follow-up with respect to the primary efficacy end point (the first occurrence of myocardial infarction, stroke, or death from cardiovascular causes) was complete in 99.5 percent of the patients randomly assigned to receive clopidogrel and aspirin and 99.6 percent of those randomly assigned to receive placebo and aspirin. The efficacy results are shown in Table 4. With a median of 28 months of follow-up, the rate of the primary event was 6.8 percent in the clopidogrel group and 7.3 percent in the placebo group (relative risk, 0.93; 95 percent confidence interval, 0.83 to 1.05; $P=0.22$) (Fig. 1A). The rate of the principal secondary efficacy end point (the first occurrence of myocardial infarction, stroke, death from cardiovascular causes, or hospitalization for unstable angina, transient ischemic attack, or a revascularization procedure) was 16.7 percent in the clopidogrel group, as compared with 17.9 percent in the placebo group (relative risk, 0.92; 95 percent confidence interval, 0.86 to 0.995; $P=0.04$) (Fig. 1B).

clopidogrel plus aspirin and 7801 were assigned to receive placebo plus aspirin. Treatment was permanently discontinued by 20.4 percent of the patients in the clopidogrel group, as compared with 18.2 percent in the placebo group ($P<0.001$). A

SAFETY END POINTS

The rate of the primary safety end point (severe bleeding according to the GUSTO definition) was 1.7 percent in the clopidogrel group and 1.3 percent in the placebo group (relative risk, 1.25; 95 percent confidence interval, 0.97 to 1.61; $P=0.09$). The rate of moderate bleeding was 2.1 percent in the clopidogrel group, as compared with 1.3 percent in the placebo group (relative risk, 1.62; 95 percent confidence interval, 1.27 to 2.08; $P<0.001$). The rate of intracranial hemorrhage was similar in the two treatment groups (Table 4).

There was one documented nonfatal case of thrombotic thrombocytopenic purpura among the clopidogrel-treated patients; this patient died one month later from end-stage chronic obstructive pulmonary disease. No other serious adverse events were reported.

SUBGROUP ANALYSES

Several prespecified subgroup analyses classified patients according to their criteria for enrollment (Fig. 2). Patients who were enrolled because they had documented cardiovascular disease were designated "symptomatic," whereas those who were enrolled because they had multiple atherothrombotic risk factors without documented cardiovascular disease were designated "asymptomatic." (Some of the latter patients had a reported history of cardiovascular events, including 10.4 percent with a prior myocardial infarction, 5.8 percent with a prior stroke, 5.2 percent with a prior transient ischemic attack, 7.7 percent who had undergone a percutaneous coronary intervention, and 9.8 percent who had undergone coronary-artery bypass grafting, although they did not meet the inclusion criteria for established cardiovascular disease as outlined in Table 1.)

Among the 3284 asymptomatic patients, there was a 20 percent relative increase in the rate of primary events with clopidogrel (6.6 percent, vs. 5.5 percent with placebo; $P=0.20$), whereas among the 12,153 symptomatic patients, there was a marginally significant reduction in the primary end point with clopidogrel (6.9 percent, vs. 7.9 percent with placebo; relative risk, 0.88; 95 percent confidence interval, 0.77 to 0.998; $P=0.046$). The interaction term for this analysis, when the differential treatment response in asymptomatic and symptomatic patients was examined, was marginally significant ($P=0.045$).

In the subgroup of asymptomatic patients, there

Table 3. Concomitant Medications.*

Medication	Clopidogrel plus Aspirin (N=7802)	Placebo plus Aspirin (N=7801)
	no. of patients (%)	
Aspirin	7775 (99.7)	7777 (99.7)
Study drug	7750 (99.3)	7760 (99.5)
Open-label clopidogrel	773 (9.9)	814 (10.4)
Diuretics	3757 (48.2)	3671 (47.1)
Nitrates	1812 (23.2)	1877 (24.1)
Calcium antagonists	2866 (36.7)	2879 (36.9)
Beta-blockers	4292 (55.0)	4344 (55.7)
Angiotensin II-receptor blockers	1990 (25.5)	2020 (25.9)
Ramipril	1387 (17.8)	1424 (18.3)
Other angiotensin-converting-enzyme inhibitors	3607 (46.2)	3612 (46.3)
Other antihypertensive agents	966 (12.4)	968 (12.4)
Statins	5991 (76.8)	6001 (76.9)
Atorvastatin	2777 (35.6)	2808 (36.0)
Simvastatin	2672 (34.2)	2695 (34.5)
Pravastatin	976 (12.5)	953 (12.2)
Fluvastatin	260 (3.3)	234 (3.0)
Lovastatin	273 (3.5)	283 (3.6)
Other statins	474 (6.1)	458 (5.9)
Other lipid-lowering agents	1114 (14.3)	1094 (14.0)
Fibrates	678 (8.7)	654 (8.4)
Binding resins	338 (4.3)	313 (4.0)
Nicotinic acid	277 (3.6)	262 (3.4)
Antidiabetic medications	3259 (41.8)	3237 (41.5)
Insulin	1360 (17.4)	1334 (17.1)
Thiazolidinediones	624 (8.0)	634 (8.1)
Other oral hypoglycemic agents	2677 (34.3)	2678 (34.3)

* These values indicate the maximal frequency of use of each agent at any time during the trial (assessed at baseline and at every follow-up visit).

was a significant increase in the rate of death from all causes among the patients assigned to clopidogrel plus aspirin as compared with those assigned to placebo plus aspirin (5.4 percent vs. 3.8 percent, $P=0.04$) as well as an increase in the rate of death from cardiovascular causes among those assigned to clopidogrel (3.9 percent vs. 2.2 percent, respectively; $P=0.01$). In contrast, clopidogrel had no significant effect on death from cardiovascular causes in the symptomatic subgroup.

The rates of GUSTO-defined severe bleeding among the asymptomatic patients were 2.0 percent

Table 4. Composite and Individual Primary and Secondary End Points.

End Point	Clopidogrel plus Aspirin (N=7802)	Placebo plus Aspirin (N=7801)	Relative Risk (95% CI)*	P Value
	no. (%)			
Efficacy end points				
Primary efficacy end point	534 (6.8)	573 (7.3)	0.93 (0.83–1.05)	0.22
Death from any cause	371 (4.8)	374 (4.8)	0.99 (0.86–1.14)	0.90
Death from cardiovascular causes	238 (3.1)	229 (2.9)	1.04 (0.87–1.25)	0.68
Myocardial infarction (nonfatal)	146 (1.9)	155 (2.0)	0.94 (0.75–1.18)	0.59
Ischemic stroke (nonfatal)	132 (1.7)	163 (2.1)	0.81 (0.64–1.02)	0.07
Stroke (nonfatal)	150 (1.9)	189 (2.4)	0.79 (0.64–0.98)	0.03
Secondary efficacy end point†	1301 (16.7)	1395 (17.9)	0.92 (0.86–0.995)	0.04
Hospitalization for unstable angina, transient ischemic attack, or revascularization	866 (11.1)	957 (12.3)	0.90 (0.82–0.98)	0.02
Safety end points				
Severe bleeding	130 (1.7)	104 (1.3)	1.25 (0.97–1.61)	0.09
Fatal bleeding	26 (0.3)	17 (0.2)	1.53 (0.83–2.82)	0.17
Primary intracranial hemorrhage	26 (0.3)	27 (0.3)	0.96 (0.56–1.65)	0.89
Moderate bleeding	164 (2.1)	101 (1.3)	1.62 (1.27–2.08)	<0.001

* CI denotes confidence interval.

† The secondary efficacy end point was the first occurrence of myocardial infarction, stroke, death from cardiovascular causes, or hospitalization for unstable angina, a transient ischemic attack, or a revascularization procedure (coronary, cerebral, or peripheral).

with clopidogrel and 1.2 percent with placebo ($P=0.07$); the corresponding rates among the symptomatic patients were 1.6 percent and 1.4 percent ($P=0.39$). Although both these differences favored the placebo group, neither was significant. The rates of GUSTO-defined moderate bleeding among asymptomatic patients were increased (2.2 percent with clopidogrel and 1.4 percent with placebo, $P=0.08$), as were the rates of moderate bleeding among symptomatic patients (2.1 percent and 1.3 percent, respectively; $P<0.001$). Again, both differences favored the placebo group, but this difference was significant only among the symptomatic patients.

DISCUSSION

In this trial of patients with established atherothrombotic disease or at high risk for such disease, there was no significant benefit associated with clopidogrel plus aspirin as compared with placebo plus aspirin in reducing the incidence of the primary end point of myocardial infarction,

stroke, or death from cardiovascular causes. There was a moderate, though significant, benefit in reducing the secondary composite end point of myocardial infarction, stroke, death from cardiovascular causes, or hospitalization for unstable angina, transient ischemic attack, or revascularization.

The rate of severe bleeding was not significantly greater with clopidogrel than with placebo, but a trend prompting concern was noted, and clopidogrel was associated with a significant increase in the rate of moderate bleeding. A total of 94 ischemic (secondary) end points were prevented with clopidogrel, at a cost of 93 moderate or severe bleeding events.

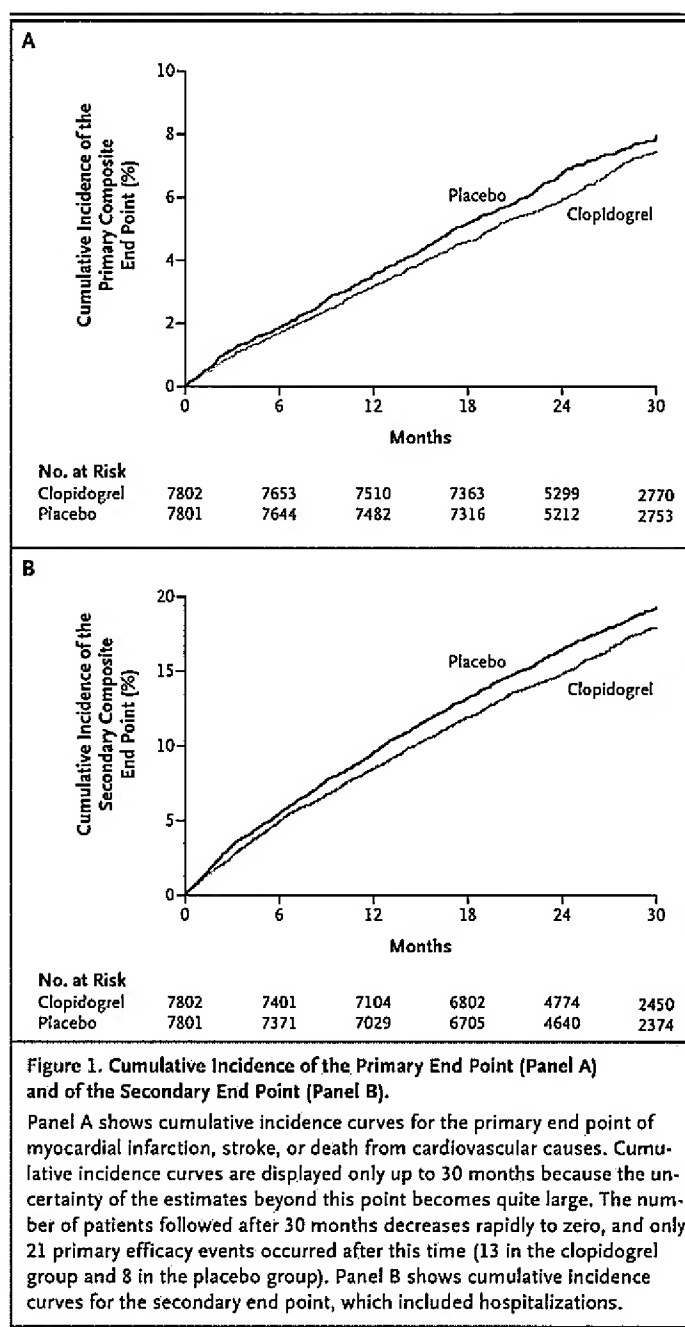
The patients in our trial received evidence-based pharmacologic treatment, with frequent use of concomitant statins, ACE inhibitors, and other background medical therapy. The incidence of the primary end point with such therapy, as predicted, was approximately 3 percent per year.

In the original, large-scale Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) trial,¹³ clopidogrel alone was found to

be superior to aspirin alone in reducing the risk of ischemic stroke, myocardial infarction, or death from vascular causes. However, there was debate as to whether P2Y₁₂-receptor blockade provided uniform benefit. Since CAPRIE, four large clinical trials have added to the body of evidence that supports the use of dual antiplatelet therapy in patients with acute coronary syndromes and in those undergoing percutaneous coronary intervention.⁶⁻⁹ CHARISMA represented the logical next step of evaluation of the potential role of this approach in a broad population of patients with established vascular disease or multiple cardiovascular risk factors.

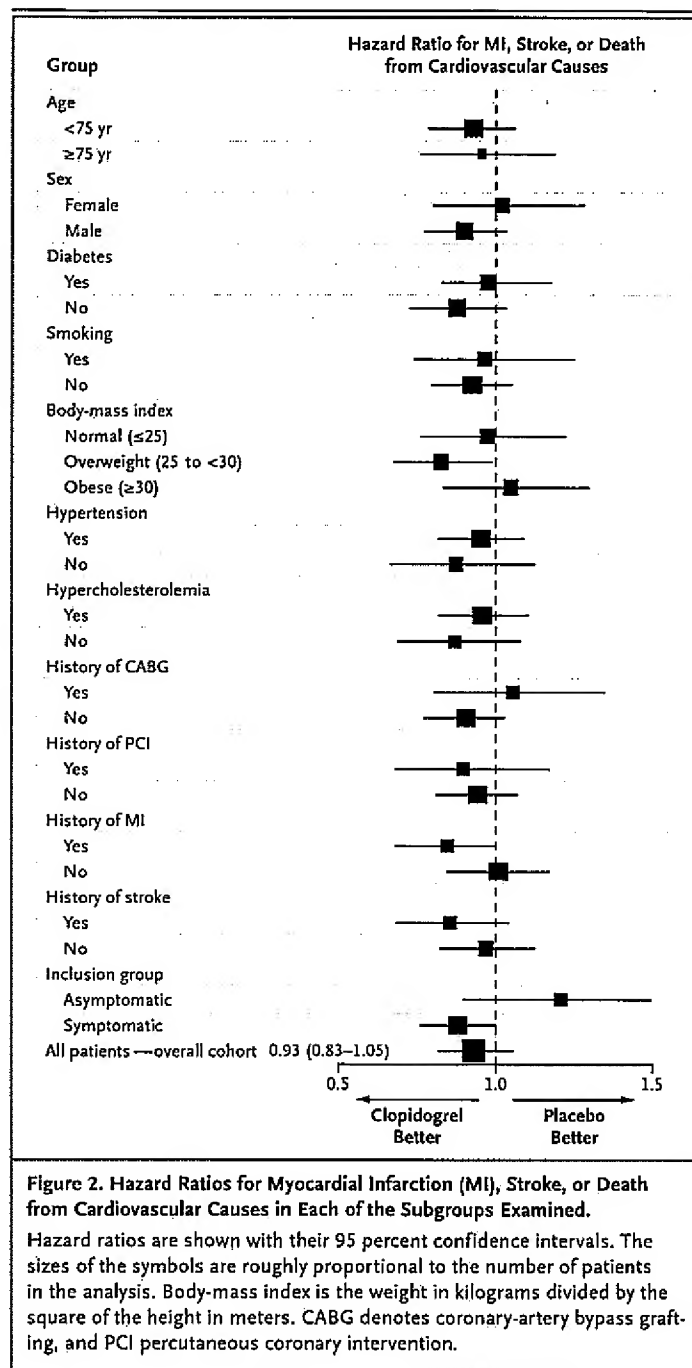
A subgroup analysis suggested that clopidogrel was beneficial with respect to the primary efficacy end point in patients who were classified as symptomatic for the purposes of the trial (i.e., who were enrolled because of a documented history of established vascular disease). However, the P value for this association and the P value for the interaction between enrollment status and therapy were only marginally significant, suggesting that this observation should be interpreted with caution, especially since this subgroup analysis was only one of several such analyses performed. Furthermore, the risk of moderate or severe bleeding in symptomatic patients was greater with clopidogrel than with placebo, although there was no significant increase in intracranial or fatal bleeding. Finally, as a practical matter, it is unclear how such a classification could be implemented clinically, since some patients in the asymptomatic subgroup actually had a history of symptoms or cardiovascular events. The issue of whether dual antiplatelet therapy is beneficial in more specific subgroups of the population of patients with atherothrombotic disease or risk will require further study.

On the other hand, the risk associated with dual antiplatelet therapy in the asymptomatic group was not anticipated. The excess fatalities in this subgroup and the heightened risk of bleeding complications suggest that we should be cautious about too quickly dismissing this unexpected finding as the play of chance. It is possible that established vascular disease represents a crude proxy for hyperactive platelets. If this concept is accepted, dual antiplatelet therapy would be anticipated to be associated with greater efficacy and a lower rate of bleeding in the subgroup of symptomatic patients. However, reduced basal platelet



activity in asymptomatic patients would be expected to be a liability, increasing the risk of bleeding complications, including possible hemorrhage into an arterial plaque. Whatever the explanation, it appears that until proven otherwise, clinicians should avoid dual antiplatelet therapy in patients without established vascular disease.

Recent studies of the genomics of myocardial



infarction and atherosclerosis have revealed a marked difference among persons in the biologic basis of disease susceptibility. Whereas multiple genes have been demonstrated to confer susceptibility to heart attack, little has been reported on the molecular determinants of atherosclerosis in

humans.¹⁴ Atherosclerosis is far more common than are vascular events such as sudden death, heart attack, and stroke, which occur in a relatively small subgroup of patients. One hypothesis that could be consistent with a benefit of dual antiplatelet therapy in symptomatic patients (those with established vascular disease) is that this group has already shown a predisposition to arterial plaque rupture, fissure, or erosion. That dual antiplatelet therapy is best used in patients who are most liable to have such arterial injury appears to be a worthy hypothesis for prospective evaluation.

In summary, the combination of clopidogrel plus aspirin was not significantly more effective than aspirin alone in reducing the rate of myocardial infarction, stroke, or death from cardiovascular causes among patients with stable cardiovascular disease or multiple cardiovascular risk factors. Furthermore, the risk of moderate-to-severe bleeding was increased. Our findings do not support the use of dual antiplatelet therapy across the broad population tested. There was a potential benefit in symptomatic patients (those with established vascular disease); this finding requires further study. Data on mortality rates suggest that dual antiplatelet therapy should not be used in patients without a history of established vascular disease.

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APPENDIX

The CHARISMA committees, national coordinators, and investigators are as follows: Executive committee: E.J. Topol (chair), K.A.A. Fox (cochair), W. Hacke (cochair), D.L. Bhatt (principal investigator), P.B. Berger, H.R. Black, W.E. Boden, P. Cacoub, E.A. Cohen, M.A. Creager, J.D. Easton, M.D. Flather, S.M. Haffner, C.W. Hamm, G.J. Hankey, S. Claiborne Johnston, K.-H. Mak, J.-L. Mas, G. Montalescot, T.A. Pearson, P.G. Steg, S.R. Steinhilber, M.A. Weber; Independent data and safety monitoring board: R.L. Frye (chair), P. Amaranco, L.M. Brass, M. Buyse, L.S. Cohen, D.L. DeMets, V. Fuster, R.G. Hart, J.R. Marler, C. McCarthy, A. Schöning; Clinical events committee: A.M. Lincoff (chair), S.J. Brener (cardiology), C.A. Sila (neurology), A. Albuquerque, G. Arountounov, D. Artemiev, B.G. Atkeson, T. Bartel, D.C.G. Basart, A. Bastos Lima, G. Belli, A.L. Bordalo e Sá, X. Bosch, G. Boysen, E.W.A. Busch, A. Cavallini, A. Chamorro Sánchez, J.H. Chiu, T. Dahl, E. Danielsson, R.B. Fathi, P. 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Exhibit 29

Coronary Heart Disease

Randomized Comparison of Prasugrel (CS-747, LY640315), a Novel Thienopyridine P2Y₁₂ Antagonist, With Clopidogrel in Percutaneous Coronary Intervention

Results of the Joint Utilization of Medications to Block Platelets Optimally (JUMBO)-TIMI 26 Trial

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Background—Despite the current standard antiplatelet regimen of aspirin and clopidogrel (with or without glycoprotein IIb/IIIa inhibitors) in percutaneous coronary intervention patients, periprocedural and postprocedural ischemic events continue to occur. Prasugrel (CS-747, LY640315), a novel potent thienopyridine P2Y₁₂ receptor antagonist, has the potential to achieve higher levels of inhibition of ADP-induced platelet aggregation than currently approved doses of clopidogrel.

Methods and Results—Joint Utilization of Medications to Block Platelets Optimally-Thrombolysis In Myocardial Infarction 26 (JUMBO-TIMI 26) was a phase 2, randomized, dose-ranging, double-blind safety trial of prasugrel versus clopidogrel in 904 patients undergoing elective or urgent percutaneous coronary intervention. Patients were randomized to either standard dosing with clopidogrel or 1 of 3 prasugrel regimens. Subjects were monitored for 30 days for bleeding and clinical events. The primary end point of the trial was clinically significant (TIMI major plus minor) non-CABG-related bleeding events in prasugrel- versus clopidogrel-treated patients. Hemorrhagic complications were infrequent, with no significant difference between patients treated with prasugrel or clopidogrel in the rate of significant bleeding (1.7% versus 1.2%; hazard ratio, 1.42; 95% CI, 0.40, 5.08). In prasugrel-treated patients, there were numerically lower incidences of the primary efficacy composite end point (30-day major adverse cardiac events) and of the secondary end points myocardial infarction, recurrent ischemia, and clinical target vessel thrombosis.

Conclusions—In this phase 2 study, which was designed to assess safety when administered at the time of percutaneous coronary intervention, prasugrel and clopidogrel both resulted in low rates of bleeding. The results of this trial serve as a foundation for the large phase 3 clinical trial designed to assess both efficacy and safety. (*Circulation*. 2005;111:3366-3373.)

Key Words: coronary disease ■ drugs ■ hemorrhage ■ platelets ■ stents

Platelet activation and aggregation play important roles in the pathogenesis of cardiac ischemic events after either spontaneous plaque disruption in acute coronary syndromes or mechanical disruption of coronary artery plaques caused by percutaneous coronary intervention (PCI).¹ The use of coronary stents has resulted in a reduced need for recurrent target vessel revascularization but an increased risk of acute and subacute thrombosis² of the instrumented vessel. Stan-

dard therapy for the prevention of thrombotic events after coronary stenting involves dual antiplatelet therapy with aspirin plus a thienopyridine.^{3,4} Thienopyridines block platelet activation and aggregation by inhibiting the P2Y₁₂ ADP receptor.⁵ Most clinical trials supporting the use of thienopyridines plus aspirin in PCI compared with aspirin alone were conducted with ticlopidine.⁶⁻⁹ However, clopidogrel has largely replaced ticlopidine for use in PCI because of better

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tolerability and a lower risk of hematologic abnormalities compared with ticlopidine.^{10,11}

Despite the widespread use of clopidogrel in patients undergoing PCI with currently available thienopyridines, several important issues remain.¹²⁻¹⁵ Data from the Clopidogrel to Reduce Events During Observation (CREDO) trial suggest that most of the acute effect seen in reducing periprocedural events with clopidogrel was limited to patients who received the drug at least 6 hours, and perhaps as many as 15 hours, before the procedure.^{16,17} As irreversible inhibitors of platelet function, the effects of thienopyridines are long-lasting, resulting in a reluctance in current clinical practice to give these agents before determining whether a patient is likely to need coronary bypass surgery.^{3,18} When bypass surgery is performed within 5 days of treatment with clopidogrel, a significant increase in major bleeding events has been observed.¹⁹ Finally, a significant variability in the response to clopidogrel among healthy subjects and patients undergoing PCI has been observed, with some individuals having minimal inhibition of ADP-induced platelet aggregation.^{15,20-22} This concept of clopidogrel resistance led to the concern that some patients may not be adequately protected from the intense platelet activation and aggregation that occur with PCI and are therefore at increased risk for thrombotic events.^{14,15,23} Because of these issues, an improved antiplatelet regimen to support PCI is desirable.

Prasugrel (CS-747, LY640315) is a novel thienopyridine antiplatelet agent that has been shown in preclinical studies to be more potent and to have a more rapid onset of action than clopidogrel.²⁴ Phase 1 studies in healthy human subjects not taking aspirin showed inhibition of platelet aggregation to be greater with a single 60-mg dose of prasugrel than a single 300-mg dose of clopidogrel²⁵ and that repeated dosing with 10 mg prasugrel showed higher inhibition of platelet aggregation than 75 mg clopidogrel.²⁶ Furthermore, there is evidence in healthy volunteers that thienopyridine resistance may be less frequent with a loading dose of 60 mg prasugrel than with 300 mg clopidogrel.²⁵ These features stimulated interest in the evaluation of prasugrel for the prevention of thrombotic events after PCI. In preparation for a future phase 3 trial designed to assess efficacy, the present study is a phase 2, dose-ranging safety trial comparing prasugrel with clopidogrel in patients undergoing PCI. The primary hypothesis for the present study was that prasugrel is as safe as clopidogrel with respect to bleeding events after PCI.

Methods

The study was conducted between April and December 2003 at 80 sites in the United States and Canada (see Appendix in the online-only Data Supplement). The protocol was approved by all relevant local institutional review boards and ethics committees, and all patients signed written informed consent forms before participation.

Study Population

To be eligible for inclusion in the trial, a patient had to (1) be a man or nonpregnant woman ≥ 18 and ≤ 75 years of age, (2) be a candidate for elective or urgent PCI with intended coronary stenting, and (3) have a native target coronary artery stenosis $>60\%$ (by visual estimation) that was thought by the operator to be amenable to stenting with ≤ 2 approved coronary stents per lesion (multilesion or multivessel stenting was acceptable if all lesions were treated in a

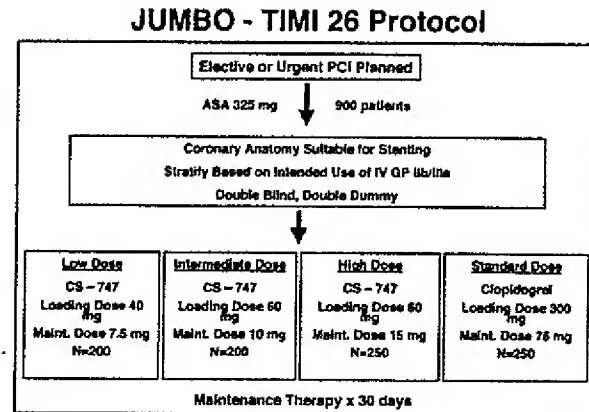


Figure 1. Protocol schema for JUMBO-TIMI 26.

single nonstaged procedure). Patients could have been enrolled before diagnostic catheterization but were randomized only if they subsequently met angiographic eligibility criteria.

Patients were excluded from the study if any of the following were present: (1) planned PCI procedure as initial treatment for an ST-elevation myocardial infarction (STEMI) or within 24 hours of fibrinolytic therapy for STEMI; (2) left main stenosis $\geq 50\%$ not protected by at least 1 patent bypass graft; (3) target lesion in a saphenous vein graft or arterial conduit graft; (4) left ventricular ejection fraction $<30\%$ by any imaging technique or New York Heart Association (NYHA) class III or IV congestive heart failure or cardiogenic shock; (5) bleeding risks, including, but not limited to, active internal bleeding, history of bleeding diathesis, recent major surgery, or significant trauma; (6) stroke within 2 years, intracranial neoplasm, AV malformation, or aneurysm; (7) uncontrolled hypertension; (8) concomitant therapies, including oral anticoagulation therapy, treatment with a thienopyridine (ticlopidine or clopidogrel) within 5 days, subcutaneous low-molecular-weight heparin within 8 hours before PCI, bivalirudin during the index admission before PCI, a proton pump inhibitor within 12 hours before PCI or in patients scheduled to receive a proton pump inhibitor after PCI (to minimize any potential inhibition of absorption of study medications), oral or intravenous H₂ antagonist within 2 hours before PCI, or any investigational drug or device within the previous 30 days.

Study Protocol

JUMBO-TIMI 26 was a multicenter, randomized, parallel-group, double-blind, double-dummy, active-comparator-controlled trial (Figure 1). After diagnostic catheterization, subjects were randomized to either prasugrel or clopidogrel if the angiographic inclusion criteria were met. The study plan was for a total of 900 subjects to be randomized to low-dose (40-mg loading dose followed by 7.5 mg daily), intermediate-dose (60-mg loading dose followed by 10 mg daily), or high-dose (60-mg loading dose followed by 15 mg daily) prasugrel or the standard dose of clopidogrel (300-mg loading dose followed by 75 mg daily) at a 4:4:5:5 ratio (200, 200, 250, 250 subjects). A 300-mg loading dose was chosen for the comparator as the standard dose of clopidogrel in clinical practice. Randomization was stratified on the basis of the investigator's intention to use a glycoprotein (GP) IIb/IIIa inhibitor during PCI. For the purpose of blinding, clopidogrel/placebo was overencapsulated without excipients. Before this study, testing determined that overencapsulated clopidogrel was bioequivalent pharmacokinetically to unencapsulated clopidogrel (according to Food and Drug Administration standards). Study doses of prasugrel were determined from prior pharmacokinetic and pharmacodynamic data.^{24,26} Blinded study drug (active prasugrel or clopidogrel and matching placebo) was administered from any time after the completion of the diagnostic angiogram to the time the patient left the recovery room after PCI. After the procedure, a complete blood count was measured daily, and

creatinine kinase-MB (CK-MB) was measured 4 to 8 hours and 12 to 24 hours after the procedure and with any ischemic symptoms. Maintenance therapy was continued for 29 to 34 days. A 30-day visit (range, 29 to 35 days) was held to assess for end points and compliance. Clinical end points were determined at hospital discharge and at the 30-day visit.

Concomitant Medications

All subjects received daily oral treatment with enteric-coated 325 mg aspirin for the duration of the study. The use of GP IIb/IIIa inhibitors was at the discretion of the treating physician. All subjects received unfractionated heparin therapy with target activated clotting times of 200 to 250 seconds for patients receiving an intravenous GP IIb/IIIa inhibitor and 250 to 300 seconds for those not receiving a GP IIb/IIIa inhibitor. At the completion of the study, decisions about continuation and dosing of antiplatelet agents were left to the discretion of the treating physician.

Trial End Points

The primary end point of the trial was non-CABG-related "significant hemorrhage" at 30 days, defined as the composite of TIMI major and minor hemorrhage. Hemorrhagic events were classified as major or minor by use of standard TIMI definitions²⁷; a clinically overt (including imaging) hemorrhage with a hemoglobin drop >5 g/dL was considered major, and a clinically overt hemorrhage with a hemoglobin drop of 3 to ≤ 5 g/dL was considered minor. A clinically overt bleeding episode with <3 g/dL drop in hemoglobin was considered minimal.²⁸ Additional safety and efficacy end points included major adverse cardiac event (MACE) components individually and in combination. MACE were defined as any one of the following, occurring through the 30-day visit after PCI: (1) death (all-cause mortality), (2) myocardial infarction (MI), (3) stroke, (4) recurrent myocardial ischemia requiring hospitalization, and (5) clinical target vessel thrombosis (CTVT) defined either as total or subtotal occlusion of the target vessel documented angiographically and occurring ≥ 2 hours after the loading dose of study drug or as urgent target vessel revascularization (any PCI or CABG) performed in response to ischemic symptoms involving the epicardial coronary artery that was the target vessel for the index procedure. Patients who did not undergo repeated coronary angiography after the initial procedure could not be determined to have CTVT. Major safety and efficacy end points were adjudicated by an independent clinical events committee that was blinded to treatment assignment.

The definition of MI, adapted from the standard American College of Cardiology/American Heart Association (ACC/AHA) definitions,^{29,30} was dependent on pre-event biomarkers and the timing of the event. In all cases, if CK-MB was greater than the upper limit of normal (ULN) at the time of the suspected event, both an increase by $\geq 50\%$ over the previous value and documentation that CK-MB was decreasing before the suspected recurrent MI were required. Within 24 hours after PCI, a subject would be considered to have had an MI with the ensuing CK-MB >3 times the ULN; within 24 hours of CABG, the threshold was CK-MB >10 times the ULN. Periprocedural MI could also be determined by either development of new, abnormal Q waves considered to be distinct from the evolution of an index MI or pathological findings of a new MI thought to be distinct from an MI in evolution before randomization. If the suspected MI was not associated with a procedure, the definition required CK-MB or cardiac troponin greater than ULN and either chest pain or ischemic discomfort lasting >20 minutes at rest or hemodynamic decompensation.

Statistical Considerations

All analyses were performed on an intent-to-treat basis of evaluable subjects. An evaluable subject was prespecified as a randomized subject who received at least the loading dose of study drug. Comparisons for the primary and secondary end points were between each prasugrel dosing group and clopidogrel using Fisher's exact test or the log-rank test. Other comparisons were done using χ^2 testing or ANOVA as appropriate. Major prespecified analyses included all

prasugrel dosing groups combined versus clopidogrel and each prasugrel dosing group individually compared with clopidogrel. A sample size of 900 subjects was chosen to provide at least 80% power to detect a 2.5-fold increase in the risk of significant non-CABG-associated bleeding that was estimated from previous studies to occur in 5% to 7.5% of clopidogrel patients.^{10,31} Secondary end points should be considered exploratory because the trial was not designed or powered to formally test these end points. Primary analyses were performed by an independent statistician at the contract research organization (Parxel, International) and verified by the sponsor and the TIMI Study Group independently. The TIMI Study Group had possession of and full access to all databases used for the analysis of the trial.

Results

Of the 905 patients randomized, 904 evaluable patients received at least 1 dose of study drug as follows: low-dose prasugrel, 199; intermediate-dose prasugrel, 200; high-dose prasugrel, 251; and clopidogrel, 254. A total of 848 patients (93.7%) completed the protocol; 53 (6%) discontinued for adverse events, personal decision, protocol violations, or physician decision; and 3 (0.3%) were lost to follow-up. There were no statistically significant differences in reasons for discontinuation from the trial among treatment groups.

Baseline and Procedural Characteristics

The baseline characteristics (Table 1) were balanced, with no significant differences between prasugrel- and clopidogrel-treated patients. Most patients (77%) were men; the median age was 60 years; and diabetes was frequent (27%). Unstable angina or NSTEMI was present in 40% of patients before PCI. Physician investigators elected to use GP IIb/IIIa inhibitors in 71% of patients.

As would be expected from the study design, nearly all patients underwent a PCI (99%), with 99% of patients who had PCI receiving at least 1 intracoronary stent. Multiple (≥ 2) stents were used in 35%. At least 1 drug-eluting stent was used in 54% of subjects. These procedural characteristics were well balanced among treatment groups.

Safety

In all groups combined, bleeding rates were low; 0.7% of patients experienced major bleeding, 1.1% experienced minor bleeding, and 2.4% experienced minimal bleeding. As would be expected in a trial of PCI, most of the bleeding episodes were related to instrumentation (68%), and the most frequent site of bleeding was the vascular access site. Most overall bleeding events (76%), including 4 of the 6 major hemorrhages, occurred during the index hospitalization. An intracranial hemorrhage (subdural hematoma) occurred in 1 patient (0.1%).

Major safety end points are summarized in Table 2. When examined by treatment group, there were low rates of major bleeding for all treatment groups (0.5% for prasugrel compared with 0.8% for clopidogrel). There was a higher incidence of but no statistically significant difference between the prasugrel groups individually or in combination compared with clopidogrel for the primary safety end point of the combination of TIMI major and minor non-CABG-related hemorrhage (1.7% versus 1.2%; hazard ratio [HR], 1.42; 95% CI, 0.40 to 5.08; Figure 2). Transfusion rates were low, with

TABLE 1. Baseline Characteristics

	Prasugrel Dose, mg (Loading/Maintenance)				Clopidogrel Dose, mg (Loading/Maintenance)	P
	40/7.5	60/10	60/15	All	300/75	
No.	199	200	251	650	254	
Age <65 y, %	65	76	74	72	77	0.12
Age (median), y	60	59	59	59	58	0.10
BMI (median), kg/m ²	29.4	29.5	29.8	29.6	29.4	0.68
White, %	91	90	90	90	94	0.13
Female, %	24	25	21	23	23	0.92
Smoker, %	21	25	24	23	31	0.07
Diabetes mellitus, %	29	25	29	27	25	0.43
Prior aspirin, %	80	73	77	77	77	0.42
ST-segment depression, %	14	12	12	12	12	0.84
GP IIb/IIIa use, %	70	69	69	69	69	0.87
Mean TIMI risk score (SD)	2.4 (1.2)	2.2 (1.1)	2.4 (1.1)	2.3 (1.1)	2.4 (1.1)	0.46
TIMI risk score ≤ 2 , %	50.3	63.0	54.2	55.7	49.6	0.18

BMI indicates body mass index. All characteristics are expressed as percents unless otherwise noted. Probability values are for the comparison of the combined prasugrel group vs clopidogrel.

0.9% of prasugrel-treated subjects and 1.1% of clopidogrel-treated patients receiving transfusion of at least 1 U of packed red blood cells. All treatment groups had significant hemorrhage rates lower than that expected from historical control subjects (5% to 7.5% major plus minor and 2% to 2.5% major).^{10,31} There was, however, numerically more minimal bleeding in the high-dose prasugrel group (3.6%) compared with the low-dose (2.0%) and intermediate-dose (1.5%) groups and the clopidogrel group (2.4%). Although most bleeding events were in-hospital for all groups, the high-dose prasugrel group had numerically increased postdischarge minimal bleeding episodes (1.2%) compared with other prasugrel dosing groups (0.5% for each).

Efficacy

There was a lower incidence of but no statistically significant difference in MACE (Figure 3A) in the overall prasugrel group (7.2%) compared with the clopidogrel group (9.4%; $P=0.26$; HR, 0.76; 95% CI, 0.46 to 1.24).

Major efficacy end points are summarized in Table 2. The incidence of MI was numerically but nonsignificantly less frequent in the prasugrel group compared with the clopidogrel group (Table 2). When MI was analyzed by higher CK-MB cutoffs in a post hoc analysis, a trend toward greater reductions in larger infarctions occurred in the prasugrel group (Figure 4). Similarly, numerically lower rates of CTVT and recurrent ischemia were seen in prasugrel-treated patients (Table 2).

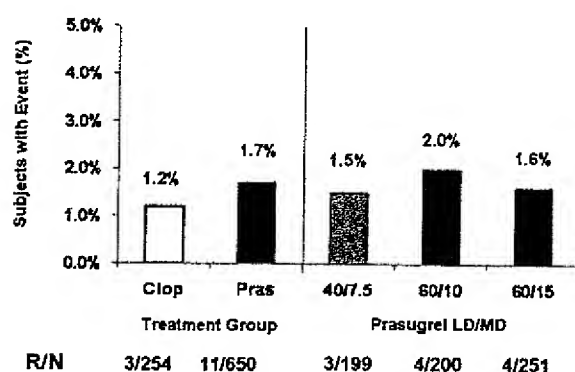
TABLE 2. Major Safety (Bleeding) and Efficacy End Points

Event	Prasugrel, n (%) (n=650)	Clopidogrel, n (%) (n=254)	P	HR (95% CI)
Bleeding				
Non-CABG TIMI major+minor	11 (1.7)	3 (1.2)	0.590	1.42 (0.40–5.08)
Non-CABG TIMI major	3 (0.5)	2 (0.8)	0.544	0.58 (0.10–3.46)
Non-CABG TIMI major+minor+minimal	27 (4.2)	9 (3.5)	0.685	1.17 (0.55–2.48)
Efficacy events				
MACE	47 (7.2)	24 (9.4)	0.260	0.76 (0.46–1.24)
Death	3 (0.5)	0	0.278	...
Stroke	3 (0.5)	0	0.278	...
MI	37 (5.7)	20 (7.9)	0.226	0.72 (0.42–1.24)
Recurrent ischemia	6 (0.9)	4 (1.6)	0.391	0.58 (0.16–2.05)
Severe ischemia	9 (1.7)	11 (3.5)	0.086	0.47 (0.2–1.14)
CTVT	4 (0.6)	6 (2.4)	0.024	0.26 (0.07–0.92)
Death/MI	40 (6.2)	20 (7.9)	0.349	0.78 (0.46–1.33)
Death/MI/CTVT	41 (6.3)	24 (9.4)	0.101	0.66 (0.40–1.10)

The trial primary end point was non-CABG-related TIMI major plus minor bleeding. Primary safety and efficacy end points are in bold. Recurrent ischemia required rehospitalization. Severe ischemia included patients for whom hospitalization was prolonged as a result of an ischemic episode. HR was not calculable for death and stroke because of zero cell in the clopidogrel group.

*Log-rank probability value.

A. Significant Non-CABG Bleeding (30 d) (TIMI Major + Minor)



B. TIMI Major Non-CABG Bleeding (30 d)

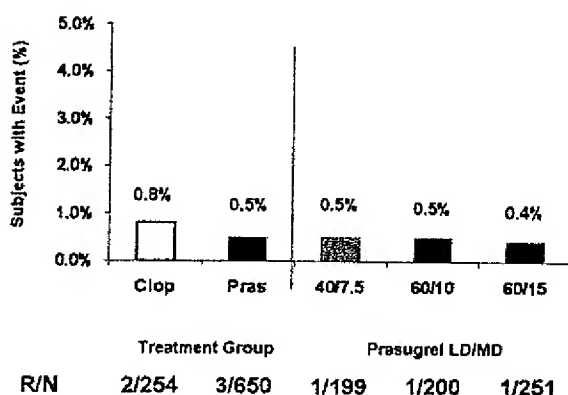


Figure 2. A, Significant bleeding. B, Major bleeding. Numbers above bar indicate percent of subjects experiencing event through 30 days of follow-up. Numbers below bars indicate number of subjects experiencing event/number at risk (R/N). LD/MD indicates loading dose/maintenance dose; 40/7.5, prasugrel 40-mg loading dose, 7.5-mg maintenance dose; 60/10, prasugrel 60-mg loading dose, 10-mg maintenance dose; 60/15, prasugrel 60-mg loading dose, 15-mg maintenance dose; Pras, all patients randomized to prasugrel; and cllop, all patients randomized to clopidogrel.

Three deaths were observed in the high-dose prasugrel group (1.2%), whereas no deaths were seen in the other treatment arms. One patient was randomized, received only a loading dose, and died of complications from an elective coronary artery bypass surgery (sepsis). A second patient had a witnessed sudden death while exercising; an autopsy showed no evidence of stent thrombosis or new ischemic event. The third death was not witnessed, and no autopsy was performed. There was no statistically significant difference in death among the prasugrel groups or between all prasugrel groups and the clopidogrel group (Table 2).

Two strokes were observed in the intermediate-dose prasugrel group (1.0%) and were nonhemorrhagic. One stroke was seen in the high-dose prasugrel group (0.4%) and was

judged to be hemorrhagic. This patient had a CT scan showing a small subdural hematoma. There were no episodes of intraparenchymal or epidural hemorrhage. There was no statistically significant difference in this end point among the prasugrel groups or between the combined prasugrel group and the clopidogrel group (Table 2).

Subgroups

Separate analyses were performed on the basis of the use of GP IIb/IIIa inhibitor, gender, age, smoking status, prior aspirin use, urgent versus elective PCI, and indication for PCI. There were no significant interactions between subgroup and treatment effect.

Discussion

This is the first report of the use of a novel thienopyridine antiplatelet agent, prasugrel (CS-747, LY640315), in patients undergoing elective or urgent PCI. This trial was designed to evaluate ranges of both loading and maintenance doses of prasugrel compared with standard therapy with clopidogrel. Bleeding rates for all treatment groups were lower than expected for clopidogrel plus aspirin from prior PCI trials.^{10,17,31} There was a suggestion of an increase in TIMI minimal bleeding in the postdischarge period with the highest dose of prasugrel compared with all other treatment arms. This suggests a clinically meaningful dose-response relationship with this compound.

This trial was designed as a phase 2 safety study. The primary goal was to assess the bleeding risk associated with prasugrel and the feasibility of a phase 3 trial. It was not designed or powered to detect clinical efficacy. Therefore, with 904 subjects in 4 treatment arms (including an active comparator), the study did not have statistical power to detect clinically meaningful differences in efficacy end points. Accordingly, efficacy results should be interpreted with caution. Point estimates for selected ischemic end points were lower in the prasugrel-treated patients. However, except for CTFT, these events failed to meet statistical significance and formally should not be considered evidence of being different.

In aggregate, these data showed that treatment with prasugrel resulted in acceptable levels of bleeding with contemporary PCI practices; there were low rates of major bleeding, significant (major plus minor) bleeding, and transfusions. There were nonsignificantly higher rates of minor and minimal bleeding in patients treated with prasugrel, especially at the highest dose studied. In contrast, there were nonsignificantly lower rates of ischemic events after PCI when patients were treated with prasugrel compared with clopidogrel. This combination of features warrants further study.

If a similar magnitude of reduction in efficacy end points seen in this trial can be corroborated in a larger trial powered to detect clinical efficacy differences with acceptable safety, this would constitute an improvement over the current standard of care. Although the present trial was not designed to assess mechanisms of action, multiple features of prasugrel could explain these putative differences, including increased level of platelet inhibition,²⁶ and/or diminished interpatient

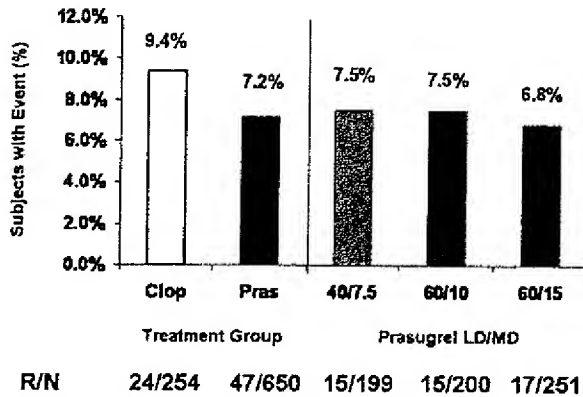
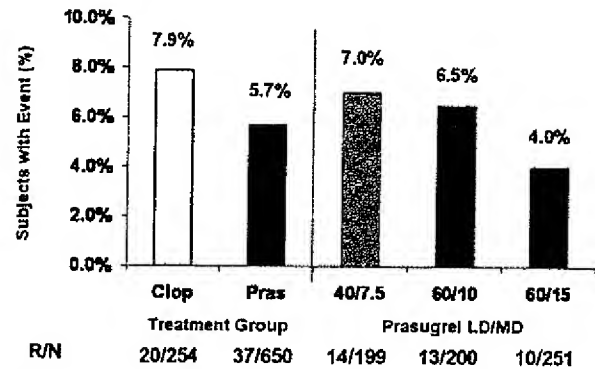
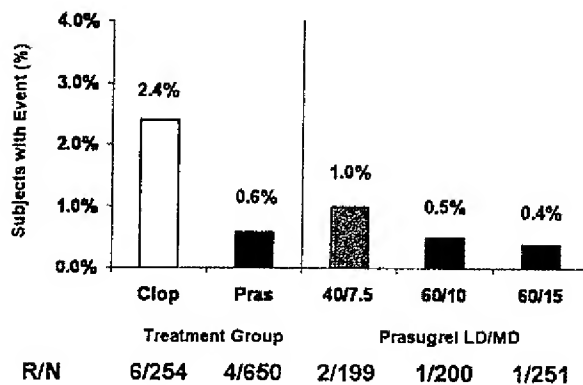
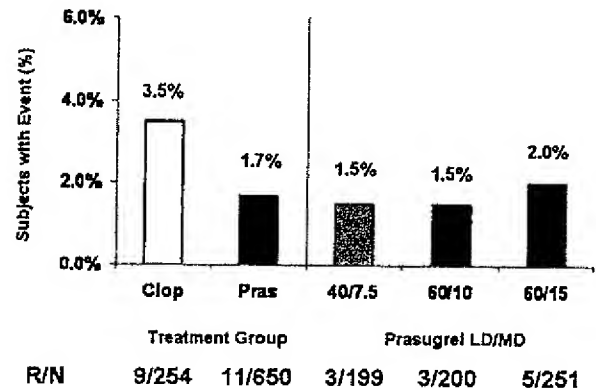
A. MACE at 30 d**B. MI at 30 d****C. CTVT at 30 d****D. Severe Ischemia at 30 d**

Figure 3. Major efficacy end points through 30 days. A, MACE. B, MI. C, CTVT. D, Severe ischemia (requiring rehospitalization or prolonging ongoing hospitalization). Numbers above bar indicate percent of subjects experiencing event through 30 days of follow-up. Numbers below bars indicate number of subjects experiencing event/number at risk (R/N). Abbreviations as in Figure 2.

variability of antiplatelet effect compared with standard clopidogrel therapy.²⁵

Study Limitations

The trial was designed as a safety trial with a primary end point comparing bleeding rates. Bleeding rates in the control arm were lower than expected, resulting in reduced power for the primary safety end point. Although the rates of bleeding were also low in the prasugrel-treated patients, one cannot exclude a moderate increase in bleeding with prasugrel. The trial was not intended to be powered to specifically examine efficacy end points. As a result, the CIs around the estimates of ischemic event reductions are wide, and the magnitude of the decrease in event rates should be interpreted with caution. However, the biological plausibility of the enhanced antiplatelet effect and the appearance of a dose-response relationship with prasugrel provide support that these observations could be clinically meaningful. To the best of our knowledge,

no adequately powered study has compared a 300-mg loading dose with higher loading doses of clopidogrel or clopidogrel pretreatment with administration of loading doses of clopidogrel during PCI with clinical end points, but some operators have adopted these practices on the basis of mechanistic information. The design of JUMBO-TIMI 26 does not allow for the assessment of prasugrel compared with higher doses of clopidogrel or the effects of longer durations of study drug pretreatment. Information about the safety of prasugrel in populations excluded from this trial (including the elderly or patients undergoing primary PCI for STEMI) cannot be determined from this trial and need to be clarified in future studies.

Conclusions

Thienopyridine antiplatelet agents are an important component of adjunctive therapy for PCI. Prasugrel is a novel, thienopyridine P2Y₁₂ antagonist that can achieve more rapid

CK-MB Elevation Level

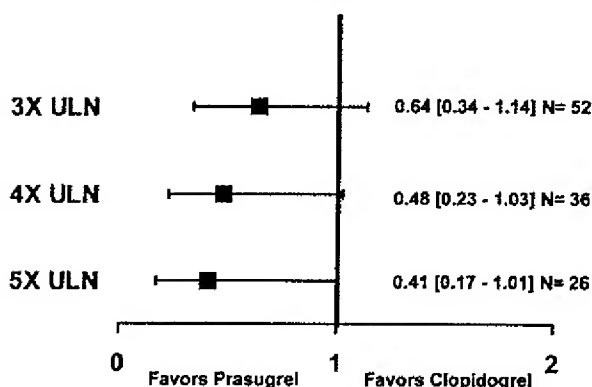


Figure 4. Hazard ratio and CIs for MI at 30 days using increasingly stringent CK-MB cutoffs. The notations 3X, 4X, and 5X indicate 3, 4, or 5 times ULN CK-MB. Number in parentheses notes number of MIs meeting this definition.

onset and higher levels of inhibition of platelet aggregation. In this study, designed to assess safety, prasugrel and clopidogrel, when administered at the time of PCI, resulted in low rates of bleeding, although modest increases associated with prasugrel cannot be excluded given the low power of the study resulting from lower-than-expected bleeding rates in both treatment groups. The results of this trial serve as a foundation for a large phase 3 clinical trial, the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel (TRITON)-TIMI 38, designed to assess both efficacy and safety.

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Disclosure

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Exhibit 30

Cardiovascular death, nonfatal MI, and nonfatal stroke	8.5	6.5	0.68 (0.54-0.87)
Cardiovascular death, nonfatal MI, and TVR	8.8	6.7	0.75 (0.59-0.96)
Cardiovascular death and MI	8.8	6.2	0.70 (0.55-0.90)
Cardiovascular death	2.4	1.4	0.61 (0.37-1.00)
MI	7.0	4.9	0.70 (0.53-0.92)
TLR	1.9	1.3	0.66 (0.39-1.14)
Stroke	0.9	0.4	0.43 (0.18-1.06)
Stent thrombosis	2.4	1.2	0.49 (0.28-0.84)
TIMI major bleeding unrelated to CABG surgery	1.3	1.0	0.74 (0.38-1.38)

Major efficacy and safety end points at 15 months

End point	Clopidogrel (%)	Prasugrel (%)	Hazard ratio (95% CI)
Cardiovascular death, nonfatal MI, and nonfatal stroke	12.4	10.0	0.79 (0.65-0.97)
Cardiovascular death, nonfatal MI, and TVR	12.0	9.8	0.79 (0.65-0.97)
Cardiovascular death and MI	11.6	8.8	0.75 (0.61-0.93)
Cardiovascular death	3.4	2.4	0.74 (0.50-1.09)
MI	8.0	6.8	0.76 (0.60-0.95)
TLR	3.2	2.2	0.70 (0.46-1.06)
Stroke	1.5	1.6	1.03 (0.60-1.70)
Stent thrombosis	2.8	1.6	0.58 (0.36-0.93)
TIMI major bleeding unrelated to CABG surgery	2.1	2.4	1.11 (0.70-1.77)

TVR=target vessel revascularization

TLR=target lesion revascularization

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In contrast with the overall study, there was no increased bleeding in the STEMI patients treated with prasugrel compared with those randomized to clopidogrel.

Interestingly, a post hoc analysis revealed that ischemic event rates at 15 months were significantly lower with prasugrel only in STEMI patients with an anterior MI. In STEMI patients with nonanterior MI, treatment effects did not differ for the primary endpoint. The test for heterogeneity of the effect of prasugrel was significant ($p=0.0053$).

"As is the case with many adjunctive therapies in infarct treatment, the highest-risk individuals tend to gain the most," said Pinto. "In this case, the anterior MI patients and diabetics gain the most. Those are the higher-risk individuals. People where you might not be getting as much bang for your buck but might still have the penalty of bleeding, include nonanterior MI patients, where there is not a clear signal of benefit."

Pinto told *heartwire* that TRITON is reflective of the general population, where 40% of infarctions are anterior MIs. This absence of benefit in the nonanterior group "should give a clinician pause," he said.

Also commenting on the TRITON analysis for *heartwire*, Dr Paul Gurbel (Sinal Hospital, Baltimore, MD) said the findings go along with the main TRITON results but added, as did investigators, that the study was not prospectively designed or powered to test the superiority of prasugrel over clopidogrel in STEMI patients. Commenting on the infarct location issue, Gurbel said that it's possible that nonanterior MIs might have been clinically silent, whereas a recurrent event in the left anterior descending artery, for example, is likely to be captured.

Regarding the lack of bleeding risk observed in STEMI patients, he said the results "are not what you'd expect" considering the amount of GP IIb/IIIa inhibitor use. Also, he noted that the rate of non-CABG TIMI major bleeding was greater in STEMI patients than in the overall cohort—up from 1.8% to 2.1% in the STEMI cohort—but was unchanged with prasugrel, another finding that is unexpected, given that prasugrel is a more potent antiplatelet agent.

"I don't think you can say that you don't have a bleeding hazard with prasugrel in STEMI patients," said Gurbel. "I think that's the wrong conclusion. I don't think the investigators are trying to say that either. I think we need to be cautious about any interpretation of these data."

Limitations of the trial

In an editorial accompanying the published study [2], Dr Gregg Stone (Columbia University, New York) writes that the TRITON study shows that additional freedom from ischemic events is possible with more powerful platelet inhibition in acute coronary syndrome (ACS) patients undergoing PCI. Also, the study "provides a tantalizing glimpse that balancing ischemic and hemorrhagic risk through careful selection of patients and personalized pharmacotherapy should result in improved outcomes for patients with cardiovascular disease."

Stone notes several limitations of the TRITON study, the first being the dose of the comparator drug. In the study, prasugrel was compared with a 300-mg loading dose of clopidogrel rather than the more potent 600-mg dose, the current standard of care for primary PCI. He also notes that STEMI patients enrolled in the study between 12 hours and 14 days after symptom onset, designated secondary PCI, likely did not receive the full benefit of clopidogrel because of inadequate preloading. Overall, 72% of patients in the clopidogrel arm received the study drug during PCI, whereas just

27% were preloaded within the allocated 24 hours prior to the procedure.

"These limitations are not idle academic musings," writes Stone. "The size of the 30-day reduction in major adverse cardiovascular events in TRITON with prasugrel was similar to that seen with adequate clopidogrel loading compared with placebo in the PCI-CURE and PCI-CLARITY studies."

Speaking with *heartwire*, Bhatt said the issues raised by Stone have validity.

"What if we used the 600-mg loading dose, or what if we pretreated? Would the relative benefits be the same?" asked Bhatt. "There'd probably be less benefit, but we know there are people who would benefit from prasugrel, because even with the higher doses of clopidogrel there are different SNPs that predispose to a lower clopidogrel effect. So even if the trials had been done with different doses or preloading, there'd probably be some differential in favor of prasugrel, but I do think the differential would be attenuated."

Whether the benefit would be worth the cost in bleeding is unknown, said Bhatt. "You have to do those trials, which are probably never going to be done on a large scale," he said. "We won't have a precise answer, and we'll be left trying to practice the fine art of medicine and interpret the data we have."

Earlier this week, the European Commission granted marketing approval of prasugrel for the prevention of atherothrombotic events in patients with ACS undergoing PCI. The Food and Drug Administration has yet to make a decision, but an advisory panel voted unanimously to approve the drug.

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Sources

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Your comments

STEMI patients in TRITON-TIMI 38: Prasugrel bests clopidogrel without bleeding risk

1 of 2 March 7, 2009 11:00 (EST)

Michael Cobble, M.D.

Impressive

The numbers look good/great. It definitely looks like those with anterior STEMI or diabetic STEMI would benefit (although a person could argue cabg for latter). I have frustration that all cause and cv mort rates were no better. The other issue would be bleed rates for those who must go on to have cabg.

2 of 2 March 9, 2009 01:51 (EDT)

Jack Quinlan

Not that impressive for PCI patients

A stronger blood thinner will work better at what cost? What does "at the expense of a significant increase in major bleeding, life-threatening bleeding, and fatal bleeding." mean? The bleeding rates in the Tables do not show a distinct or strong trend. Further, the lower loading dose for the clopidogrel patients might mask an important observation for stent thrombosis rates. Comparing the 30-day and 15-month figures we see that stent thrombosis has risen just 0.4% for both drugs. Does this show the low clopidogrel loading dose limitation in the results?

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Susan Frattura

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Exhibit 31

Variable Interindividual Responses to Antiplatelet Therapies – Do They Exist, Can We Measure Them, and Are They Clinically Relevant?

Insights from the GOLD (AU – Assessing Ultegra) Trial

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Key Words

Platelet(s) · Aspirin · Clopidogrel · Ticlopidine · Glycoprotein IIb/IIIa inhibitors

Abstract

Many patients suffer thrombotic events such as myocardial infarction, stroke and peripheral embolism despite therapy with recommended doses of all currently approved antiplatelet agents. Researchers have suggested that a subset of patients may be resistant to the antiplatelet effects of aspirin, and have developed substantial evidence to support this theory. The thienopyridines ticlopidine and clopidogrel and the glycoprotein IIb/IIIa inhibitors also exhibit substantial interpatient variability in the level of platelet inhibition they achieve. There are several biochemical factors that may contribute to the etiology of individual resistance to antiplatelet medications. Some studies suggest that the variability in patient responsiveness to these drugs may have clinical consequences, and data from trials evaluating clinical end points are needed to further elucidate this correlation.

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Introduction

As the role of the platelet in coronary thrombosis becomes clearer, the importance of antiplatelet strategies in acute coronary syndromes (ACS) and percutaneous coronary interventions (PCI) is gaining considerable attention. Aspirin, the first drug found to impede clot formation through its action on platelets, was discovered to profoundly improve the outcome of patients suffering an acute myocardial infarction when the ISIS-2 investigators demonstrated a 21% reduction in mortality among patients on aspirin as compared with those on placebo [1]. It has been subsequently proven that aspirin reduces the incidence of stroke, myocardial infarction and vascular death by 25% in patients with significant risk factors for vascular events [2]. The adenosine diphosphate (ADP)-blocking agents ticlopidine and clopidogrel have been shown to decrease thrombotic events in similar populations of patients to a slightly greater degree [2–5]. Recently, glycoprotein (GP) IIb/IIIa-inhibiting drugs have gained considerable attention for their use in improving outcomes with ACS and PCI and decreasing major adverse cardiac events, including 1-year mortality, following percutaneous revascularization [6–8].

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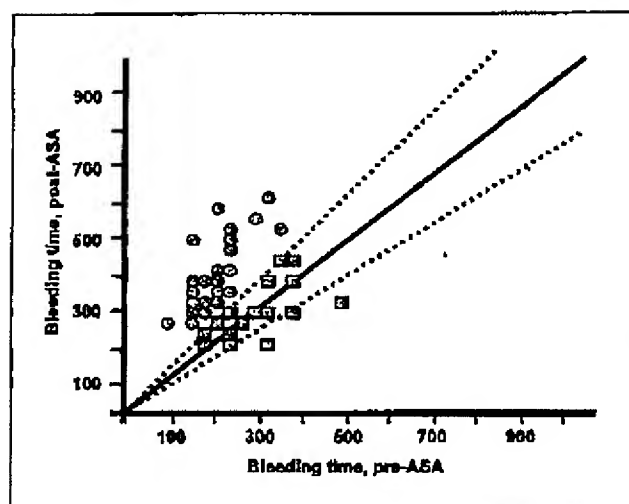


Fig. 1. Variability in bleeding time among aspirin responders (58%) and nonresponders (42%). ASA = Aspirin. The mean variation in bleeding time for responders was $58 \pm 10\%$, and for nonresponders it was $2 \pm 4\%$. Adapted from Buchanan and Brister [12].

An undesirable level of morbidity and mortality following acute coronary events persists despite our increasingly sophisticated arsenal of drugs that hinder the activation and aggregation of platelets in the face of a highly thrombotic environment. A subset of patients treated with the recommended doses of aspirin, a thienopyridine or a GP IIb/IIIa inhibitor may persist in forming new clots, thereby jeopardizing the myocardium and risking arrhythmic and mechanical complications. This brings up certain questions: Do platelet-blocking agents exhibit a degree of unpredictability in therapeutic effect? Do individuals respond differently to antiplatelet medication? Can interpatient variability be measured using platelet function assays, and does this translate into a similar variation in clinical effect and outcome? Can laboratory-guided prescribing of antiplatelet medication improve care by targeting individuals who require elevated doses of drugs or alternative treatments to achieve adequate platelet inactivation? We will attempt to answer these questions with a review of the literature and present an introduction to the GOLD study, the first trial designed to correlate measured platelet function and clinical outcomes in a cohort of patients treated with GP IIb/IIIa inhibitors.

Aspirin

Aspirin irreversibly inhibits platelet cyclooxygenase-1 (COX-1), thereby rendering platelets incapable of synthesizing thromboxane A_2 (TxA_2) for the life of the cell. TxA_2 is released by activated platelets and leads to the recruitment and eventual aggregation of additional newly activated platelets into a nascent thrombus. While aspirin exhibits other effects on the vascular milieu – such as inhibiting prostaglandin [9] and blocking the activity of nitric oxide inhibitors [10] – the effect on TxA_2 is posited to account for the principle antithrombotic effects of the drug.

Alexander et al. [11], in 1999, reported that 63.8% of patients presenting with a non-ST segment elevation ACS were actively taking aspirin. Why this subset of patients experiences a thrombotic event despite chronic antiplatelet therapy is unclear. A possible explanation arises from research that has identified a subset of the population that fails to exhibit the expected platelet inactivation with aspirin. To date, limited data implicate an association between these laboratory measurements of platelet activity and clinical outcomes, suggesting that individuals who receive inadequate platelet inhibition are at greater risk of thrombotic complications than persons who have laboratory evidence of sufficient platelet inhibition.

The concept of aspirin resistance has gained support as researchers have demonstrated significant interpatient variability in measured markers of platelet function in persons taking standard doses of the drug. Clinically, aspirin resistance is defined as the failure of aspirin to prevent thrombotic events. In an attempt to refine the boundaries of aspirin resistance, researchers have used laboratory surrogates – in the form of various platelet function assays – to estimate the prevalence of this entity at as high as 40% (fig. 1) [12].

Several problems exist with respect to such estimations.

First, there is no consensus of opinion as to the most reliable or clinically relevant marker of aspirin-induced platelet blockade. The methods employed most commonly include bleeding time [13], platelet aggregation in response to platelet activators [13–16], platelet aggregation ratio [17, 18] and flow cytometry to detect membrane GPs expressed on activated platelets [15, 19]. The absence of a single 'gold standard' makes comparisons between different studies difficult. Furthermore, only aggregation testing has been significantly linked to clinical outcome data [16, 18].

Second, in vitro aspirin responsiveness appears to differ to some degree based on the dosing used. For example,

in one study of patients undergoing coronary artery bypass surgery after 6 months of therapy with 325 mg of aspirin, 17 of 40 (42%) patients were found to have a bleeding time that failed to prolong more than 2 standard deviations and were deemed aspirin nonresponders [12]. In another arm of the same study, only 3 out of 10 healthy volunteers showed prolongation in bleeding time at a dose of 80 mg daily. When the aspirin dose was increased to 1,300 mg daily, 6 of the 7 remaining volunteers had prolonged bleeding times. The authors concluded that most volunteers who were aspirin nonresponders at the low doses of aspirin would respond to the higher dose of aspirin and suggested that the 42% aspirin resistance found in the cohort of coronary artery bypass graft patients might change with increased dosing of aspirin. The concept of a dose-dependant *in vitro* response to aspirin was documented by a trial that measured platelet aggregation in 107 patients on varying doses of aspirin for stroke prevention [14]. At an aspirin dose of 325 mg daily, inhibition of platelet aggregation was complete in 79% of patients, with 21% of the subjects exhibiting only partial blockade. Escalating the dosage to 1,300 mg in 12 persons in the latter group resulted in complete inhibition in all but 3 patients.

Clinically, however, a dose response has not been found. Dosing aspirin at 100 mg proved to be no less effective than 1,000 mg in preventing restenosis after femoropopliteal percutaneous transluminal angioplasty [20]. A meta-analysis reviewing aspirin dosing in 11 randomized, placebo-controlled trials concluded that all doses from 50 to 1,500 mg daily produced the same reduction in stroke risk (15%) in patients with a history of cerebrovascular disease [21]. In fact, some data are more suggestive of a greater clinical benefit with lower doses of aspirin. A meta-analysis of low- versus high-dose aspirin suggested a better outcome with smaller doses of the drug [22]. A recent randomized trial of aspirin dosing in patients undergoing carotid endarterectomy concluded that patients taking 325 mg or less of aspirin had fewer adverse events within 3 months than did the patients taking 650–1,300 mg of aspirin daily [23].

Third, in addition to significant interpatient variability, there may exist some degree of inpatient variability. Researchers who measured platelet aggregation in 171 stroke patients found that 154 subjects attained complete platelet inhibition on doses of aspirin varying from 325 to 1,300 mg per day [24]. Of these 154 subjects that presumably had an adequate response to aspirin, 47 (30.5%) did not maintain that effect upon repeated testing, despite fulfilling the criteria of regular compliance checks. The

results of this trial suggest that aspirin responsiveness is dynamic over time in many individuals. Such a notion was challenged by a small trial that evaluated platelet aggregation in 31 healthy, young adults and demonstrated that once platelet inhibition was achieved, it was maintained throughout the duration of the 28 days of prolonged aspirin ingestion [25]. In another small series of healthy men taking 324 mg of aspirin daily, bleeding time and platelet aggregation were found to be constant in each individual on separate assessments 30 months apart [26].

Mechanisms of Aspirin Resistance

The mechanism underlying aspirin resistance has yet to be fully elucidated, but a number of factors have been shown to cause decreased aspirin efficacy alone or in combination with other variables. Aspirin inhibits COX-1 from metabolizing arachidonic acid to the potent platelet agonist TxA_2 , but the drug has little to no effect on lipoxxygenase [12]. Lipoxxygenase converts arachidonic acid into 12-hydroxyeicosatetraenoic acid (12-HETE), a metabolite that increases platelet adhesivity. When platelet COX-1 is inhibited, platelet 12-HETE synthesis via the lipoxxygenase pathway may increase. It is conceivable that lipoxxygenase could be more prevalent or more active in persons who show resistance to aspirin. In a study by Buchanan and Brister [12], platelet 12-HETE synthesis and platelet adhesivity remained unchanged or became enhanced with aspirin therapy in the patients classified as aspirin nonresponders, whereas aspirin responders all showed decreased 12-HETE production with aspirin therapy.

Aspirin completely blocks COX-1, but its effect on COX-2 is 170 times weaker [27]. In aspirin-resistant patients, the persistent production of TxA_2 may occur as the result of unusually enhanced COX-2 activity. Furthermore, COX-2 synthesis, even in normal patients, can be rapidly induced by proinflammatory or mitogenic stimuli, including cytokines, endotoxin and growth factors [28]. Since TxA_2 needs to be blocked by 95–99% to inhibit platelet aggregation [29], even small amounts of COX-2-produced TxA_2 could feasibly result in clinically significant aspirin failure. It has been suggested that high platelet turnover can enhance platelet COX-2 expression and thereby inhibit the effect of aspirin on platelet aggregation [30]. One study found that 20% of patients with unstable angina treated with aspirin had unusually high rates of thromboxane metabolite in 6- to 8-hour urine collections, suggesting persistent thromboxane production despite aspirin therapy [31].

Aspirin is able to irreversibly acetylate the COX-1 that is present not only in platelets, but also in monocytes/macrophages and vascular endothelial cells. While this results in inhibition for the life span of the affected platelet, nucleated cells are capable of synthesizing new COX-1. The plasma half-life of aspirin is too brief (15–20 min) to suppress extra-platelet COX-1 throughout the drug-dosing interval. Indobufen, a reversible inhibitor of COX-1 with a half-life of 8 h, proved to suppress the rate of TxA₂ biosynthesis better than aspirin when given to patients with unstable angina [32]. This supports the speculation that nucleated cells in the vessel serve as a reservoir of TxA₂ synthesis.

The platelet GP complex IIb/IIIa, which acts as a receptor for fibrinogen and other adhesive molecules, is required for platelet aggregation. A polymorphism in the gene encoding GP IIIa results in the presence of two alleles in the population: PI^{A1} and PI^{A2}. The presence of the allele PI^{A2} has been suggested as a heritable risk factor for coronary artery disease [33]. In an evaluation of patients suffering myocardial infarction, researchers found a 50% incidence of the A2 allele compared with 27% in age- and sex-matched control subjects [34]. It appears that this genetic variable may also play a role in an individual's response to aspirin. One study found that aspirin therapy might be associated with elevated thrombin levels in persons with the PI^{A2} allele [35]. However, this issue is far from settled. Two other studies produced results that counter the suggestion that the PI^{A2} allele is culpable for resistance to aspirin therapy, by showing enhanced inhibition of platelet aggregation with aspirin in individuals who are heterozygous for the PI^{A2} allele [36, 37].

Kawasaki et al. [26] suggest that aspirin resistance is a function of the sensitivity of platelets to collagen. They demonstrated that the collagen concentration required to trigger platelet aggregation in aspirin nonresponders is half the concentration needed to stimulate platelet aggregation in aspirin responders. This difference was seen both with and without aspirin, and on two separate measurements performed 30 months apart.

Other mechanisms that may play a role in thrombosis despite aspirin therapy are variable rates of aspirin hydrolysis [38, 39], platelet stimulation via shear stress, ADP and endothelial prostacyclin [28] and variability in platelet aggregation related to a polymorphism of the platelet arginine vasopressin V₁ receptor [40]. Tobacco use [41], hyperlipidemia [42], testosterone level [43] and exercise [44] also affect platelet inhibition in response to aspirin.

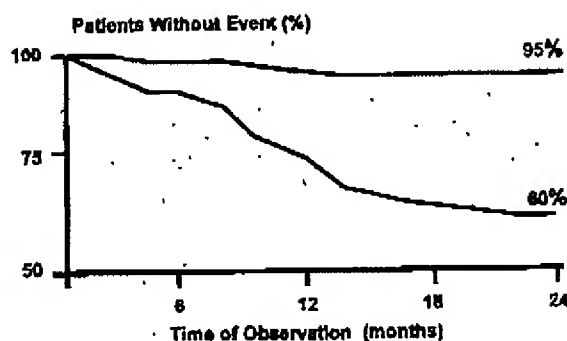


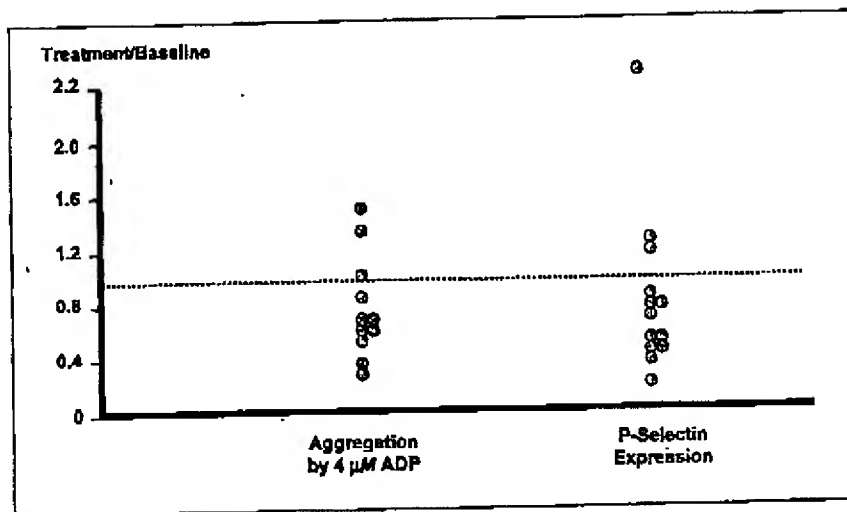
Fig. 2. Rate of major cardiac events among aspirin responders (n = 114) and nonresponders (n = 60) (p < 0.0001). Adapted from Grottemeyer et al. [18].

Clinical Relevance of Aspirin Resistance

While there are many studies demonstrating variable levels of platelet function with aspirin therapy, few trials have been performed showing that this concept translates into clinical significance. Grottemeyer et al. [18] evaluated 176 stroke victims upon their discharge from hospital and classified them as aspirin responders or nonresponders based on platelet function 12 h after a 500-mg oral dose of aspirin. All patients were treated with 1,500 mg of aspirin per day and followed for 24 months for the major end points of stroke, myocardial infarction or vascular death. Major end points were seen in only 5 of the 114 (4.4%) aspirin responders, but in 24 out of 60 (40%) nonresponders (p < 0.0001), suggesting more thromboembolic events in patients with poor platelet inhibition (fig. 2).

Another study evaluated 70 male and 30 female patients with intermittent lower extremity claudication who were undergoing percutaneous balloon angioplasty at the level of the iliaco-femoral artery [16]. The researchers measured the platelet reactivity in response to ADP and collagen after the patients were placed on aspirin at a dose of 100 mg per day. During the subsequent 18 months of clinical observation, eight patients suffered reocclusion at the site of the angioplasty. Comparisons of the aggregation results in this group revealed that restenosis occurred exclusively in male patients who failed to achieve inhibition of platelet aggregation in response to both ADP and collagen. The relative risk for reocclusion in the patients who did not respond appropriately to aspirin was 1.871 (p = 0.00093).

Fig. 3. Variability of platelet aggregation and P-selectin expression among 9 healthy volunteers treated with 250 mg of ticlopidine twice a day and 325 mg of aspirin daily for 5 days. Note the wide variation in treatment-to-baseline ratio among in vitro measurements. Adapted from Farrell et al. [15].



An ongoing trial assessing long-term cardiovascular events in patients with coronary disease has shown that 8–12% of patients taking aspirin do not achieve the therapeutic benefit of platelet inhibition, based on aggregometry [45]. The results of this study should shed light on the clinical relevance of laboratory-measured aspirin resistance.

As shown, platelet function studies reveal a significant variation in an individual's response to aspirin and suggest that a subset of the population might be resistant to the drug's protective effects against thromboembolic complications. What is less clear is whether the mechanisms that confer aspirin resistance also affect an individual's response to other antiplatelet medications.

Ticlopidine and Clopidogrel

The thienopyridines, ticlopidine (Ticlid) and clopidogrel (Plavix), irreversibly inhibit platelet aggregation by preventing ADP-mediated structural alterations in the GP IIb/IIIa receptor, thereby inhibiting platelet binding to fibrinogen [46]. Both drugs, when used chronically in the place of aspirin, have been shown to be slightly more effective than aspirin in the secondary prevention of thrombotic events [3, 5]. When dosed simultaneously, the thienopyridines and aspirin have synergistic antiplatelet effects [47].

Although not as well studied as with aspirin, interindividual variability has also been observed in platelet reactivity during treatment with this class of medication. Far-

rell et al. [15] studied platelet aggregation in healthy subjects treated with 250 mg of ticlopidine twice daily for 5 days. Substantial variation in aggregation response to ADP was seen among the patients in this cohort; 15% of specimens revealed increased aggregation with ticlopidine. Flow cytometric determination of P-selectin, a surface protein expressed on activated platelets, similarly showed a range of drug effect that varied from full platelet inhibition to little or none. Blood samples from a group of patients taking 75 mg of clopidogrel daily demonstrated a similar range of effect [48]. ADP-induced aggregation in healthy subjects on 75 mg daily showed a mean level of platelet inhibition that was within the therapeutic range on day 2 of treatment, but the variability in the group was $\pm 27\%$ from the mean (fig. 3).

Variable levels of platelet inhibition have therefore been documented among patients in these small series. Does this imply that a subset of the population will be thienopyridine nonresponders and, as seen with aspirin, exhibit ticlopidine or clopidogrel resistance? Unfortunately, no studies have been performed to assess whether patients with decreased in vitro effect from therapy with thienopyridines suffer more thrombotic complications.

GP IIb/IIIa Inhibitors

The platelet GP IIb/IIIa receptor is a platelet-specific integrin that mediates platelet aggregation, binding fibrinogen and von Willebrand factor in a common response of platelets to stimulation by all agonists. The inhibitors of

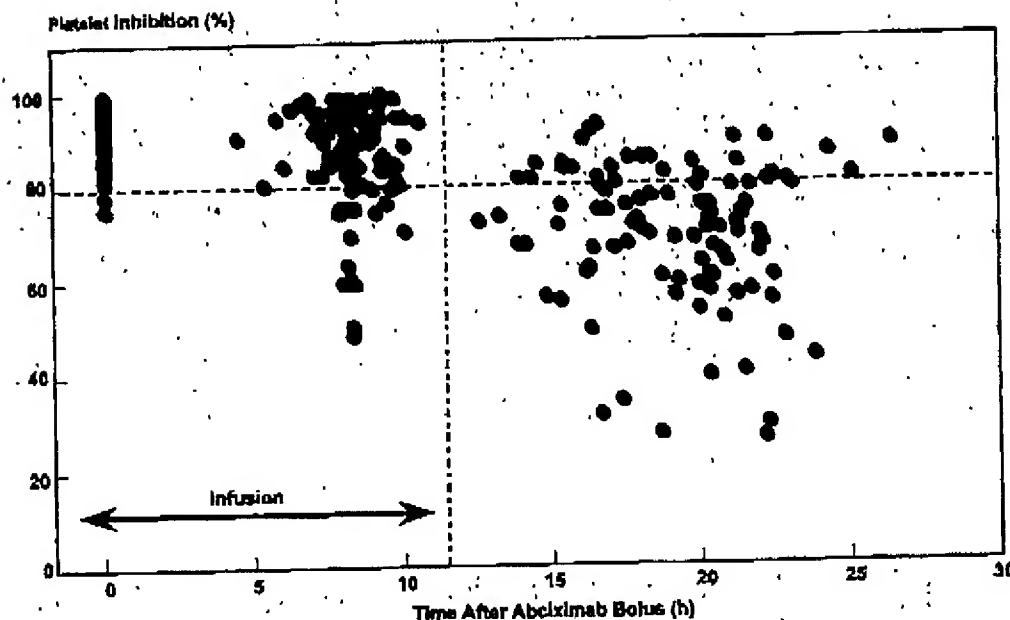


Fig. 4. Variability in platelet inhibition (as measured by aggregometry) following bolus and infusion of abciximab. At 12–20 h there is wide variation in platelet function, ranging from >80% to <30%. Adapted from Steinhubl et al. [50].

GP IIb/IIIa represent a class of drugs that compete with fibrinogen for occupancy of its platelet receptor and thereby restrict platelet aggregation. Abciximab (ReoPro), a monoclonal antibody Fab fragment, and other naturally occurring and synthetic peptide and nonpeptide antagonists of the GP IIb/IIIa receptor have been thoroughly studied and proven to limit thrombotic complications during acute coronary events and after coronary interventions.

The initial animal studies with abciximab suggested that blockade of >80% of the platelet GP IIb/IIIa receptors – with a corresponding $\geq 80\%$ inhibition of platelet aggregation – is necessary to arrest thrombosis in a thrombogenic environment [49]. In the largest study to date, platelet aggregation was measured in 97 patients receiving abciximab in conjunction with coronary angioplasty [50]. The degree of platelet inhibition was evaluated immediately after abciximab bolus (0.25 mg/kg), 8 h after beginning the 12-hour infusion (0.125 $\mu\text{g/kg/min}$) and the following day (13–26 h after the bolus). All patients but one achieved >80% platelet inhibition immediately after the infusion. Eight hours after the bolus, but still within

the infusion period, 13% of the patients had a level of platelet blockade under 80%, implying a loss of meaningful protection against thrombosis in this group. By the next morning (13–26 h after the bolus), only 29% of patients continued to have >80% inhibition of platelet activity (fig. 4). This study demonstrated a substantial variability in the capacity of abciximab to impede platelet aggregation in patients undergoing coronary angioplasty that was not predictable based on any clinical or hematological parameters, and uncovered a subset of patients that may be refractory to the antithrombotic effects of the recommended doses of abciximab. Interestingly, although this study was not designed to evaluate clinical outcomes, the investigators did find a significant increase in risk for adverse events in those patients with less than 80% platelet inhibition at 8 h.

Other studies in limited numbers of patients have similarly reflected a substantial heterogeneity in individual response to therapy with abciximab and other GP IIb/IIIa inhibitors [51–56]. One study suggested that patients with unstable angina are more refractory to the antiplatelet effects of GP IIb/IIIa blockade than patients with stable

angina [57]. The clinical impact of this heterogeneity in a large population receiving GP IIb/IIIa inhibitors is currently unknown.

The GOLD Study

The purpose of the GOLD (from the chemical symbol for gold; AU – Assessing Ultegra) study is to identify the level of platelet inhibition at several time points in patients undergoing a PCI who are being treated with a GP IIb/IIIa inhibitor, and to establish what level of inhibition is associated with the fewest thrombotic complications. The study will also determine what percentage of patients achieves this level [58]. It will prospectively evaluate the degree of platelet inhibition in 500 patients undergoing PCI who are being treated with any of the three currently approved GP IIb/IIIa inhibitors – abciximab, eptifibatide and tirofiban. In vitro platelet evaluation will be performed using the Ultegra-Rapid Platelet Function Assay (RPFA) (Accumetrics, San Diego, Calif., USA), an automated device that assesses platelet function in whole blood utilizing the ability of activated platelets to bind fibrinogen. Fibrinogen-coated polystyrene microparticles agglutinate in whole blood in proportion to the number of unblocked platelet GP IIb/IIIa receptors. Pharmacological blockade of GP IIb/IIIa receptors prevents this interaction and subsequently diminishes agglutination in proportion to the degree of receptor blockade achieved [59]. The Ultegra-RPFA has been validated against aggregometry in 120 patients undergoing PCI and treated with a GP IIb/IIIa inhibitor [60]. Enrollment in the GOLD study concluded in late 1999. Preliminary results were presented at the 2000 American College of Cardiology Meeting and demonstrated a significant correlation between the level of platelet inhibition and the occurrence of major adverse cardiac events.

Summary

The concept of variable patient response to medication is not new to clinicians. It is common to expect patients with diabetes, hyperlipidemia and hypertension to demonstrate individual nuances in their response to medications used to treat these disorders. The physician monitors the efficacy of treatment using various physical examination findings and laboratory values and makes adjustments as needed to optimize care.

Such is not the case with antiplatelet therapy. The current practice is to place all patients on the standard doses of antiplatelet agents and assume adequate protection from thrombotic complications without monitoring platelet function in each individual. With new evidence suggesting significant heterogeneity in individual responses to all antiplatelet medications, a move toward laboratory-guided platelet inhibition may be warranted.

Still, the question remains: if we identify a patient whose platelet function is not amply inhibited, what adjustment in therapy should we make to minimize thrombogenesis? Some research [12, 14, 24] suggests that aspirin resistance is at least partly a dose-dependant phenomenon and that dose escalation in targeted individuals might enhance efficacy. But even these studies found a minority of patients who failed to exhibit appropriate platelet inactivation at the maximum aspirin dose. At least one small trial has found that patients whose platelet function was insufficiently inhibited on 81 mg of aspirin demonstrated a paradoxical increase in platelet activation on higher doses [61]. Importantly, clinical trials have not uniformly supported the rationale of using higher dosing of antiplatelet medication to overcome the effect of drug resistance [20–23]. These data suggest that alternative methods of platelet blockade must be sought for patients resistant to all doses of conventional antiplatelet medication. At this point, the treatment strategy for such patients is far from perspicuous and may ultimately entail a combination of dosing changes and alternative medications.

Variable degrees of platelet blockade, based on in vitro assays of platelet function, have been consistently demonstrated in persons taking aspirin, thienopyridines and GP IIb/IIIa inhibitors. Small studies have suggested the clinical importance of this resistance to therapy in aspirin-treated patients. It appears that certain patients treated with GP IIb/IIIa inhibitors are in jeopardy of increased thrombotic complications if they fail to show an adequate response to the drug as measured in the clinical laboratory.

Despite the critical role of antiplatelet therapies in the treatment of cardiovascular diseases, there remain substantial gaps in our knowledge regarding the clinical impact of inadequate platelet blockade in persons receiving aspirin, ticlopidine, clopidogrel or the GP IIb/IIIa inhibitors. The GOLD study is the first large-scale clinical trial to identify a link between measured platelet function and clinical outcomes in patients receiving GP IIb/IIIa-inhibiting drugs in the setting of coronary intervention. Its results are the first step towards prospective treatment trials guided by individual monitoring of patient platelet response to this class of medications.

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Exhibit 32

The Lack of Augmentation by Aspirin of Inhibition of Platelet Reactivity by Ticlopidine

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A decreased threshold for platelet activation apparently contributes to the risk of cardiovascular events, such as acute myocardial infarction. To evaluate the impact of specific agents, we characterized platelet reactivity in 9 healthy subjects before and after 5 days of ingestion of 4 commonly prescribed regimens, 81 mg of aspirin daily, 325 mg of aspirin daily, ticlopidine 250 mg twice daily, and ticlopidine plus 325 mg of aspirin daily. Platelet reactivity was assessed with (1) aggregometry induced by 4 μ M adenosine diphosphate (ADP) and collagen (0.19 mg/ml) and performed in platelet-rich plasma; and (2) flow cytometric determination of ADP-induced (0.2, 0.8, and 1.5 μ M) P-selectin expression in whole blood. Because anticoagulants alter platelet reactivity, results were obtained with 3 anticoagulants, citrate, enoxaparin, or corn trypsin inhibitor (CTI, a specific inhibitor of factor XIIa without effect on other coagulation factors). Ingestion of aspirin did not alter

platelet activation as assessed with flow cytometry. Inhibition of the second phase of aggregation was seen with ADP-induced aggregation in platelet-rich plasma anticoagulated with citrate but not enoxaparin or CTI. Ingestion of ticlopidine led to inhibition of ADP-induced aggregation and P-selectin expression. Inhibition of platelet reactivity after the combination of aspirin and ticlopidine did not differ from ticlopidine alone. Marked interindividual variability in platelet reactivity was seen after ingestion of ticlopidine. The results indicate that assessment of effects of specific pharmacologic regimens with accurate and readily available assays of platelet reactivity may facilitate effective prophylaxis and treatment of high-risk subjects with antiplatelet regimens designed to optimally diminish platelet reactivity. ©1999 by Excerpta Medica, Inc.

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Increased platelet reactivity presages acute coronary syndromes in subjects with coronary disease and in otherwise healthy persons without clinical evidence of coronary disease.¹⁻¹⁰ The administration of aspirin to patients with coronary artery disease decreases mortality and reduces the subsequent incidence of myocardial infarction.²⁻⁵ Aspirin in combination with ticlopidine for 30 days decreases early closure after implantation of intracoronary stents.¹¹

We have recently described an assay in which platelet reactivity in response to physiologic concentrations of ADP is determined in whole blood with flow cytometry.¹² Because the specific anticoagulant into which blood is drawn is a determinant of platelet reactivity, we hypothesized that assessment of platelet reactivity in response to physiologic concentrations of ADP and with the use of corn trypsin inhibitor (CTI, a specific inhibitor of factor XIIa) as the anticoagulant would facilitate the accurate assessment of antiplatelet therapy on platelet reactivity. Accordingly, we characterized the effect of 4 commonly used antiplatelet regimens on ADP-induced α -granule degranulation and aggregation of platelets in blood anticoagulated with CTI, enoxaparin, or citrate. Platelet reactivity was determined before and after administration for 5

days of aspirin (81 and 325 mg daily), ticlopidine (250 mg twice daily), and aspirin (325 mg) plus ticlopidine (250 mg twice daily).

METHODS

Subjects: Nine healthy men (21 to 38 years of age) without a significant medical history participated in a protocol approved by the University of Vermont Institutional Review Board and provided written informed consent. No subject had taken aspirin or any nonsteroidal anti-inflammatory medication for at least 10 days before participation. Phlebotomy was performed by peripheral venipuncture between 8 A.M. and 11 A.M. in subjects who were not fasting. Application of tourniquets was limited to <1 minute, and venipuncture was performed with a 19-gauge butterfly needle from an antecubital vein. After discarding the first 3 ml of blood, blood was drawn into a syringe prefilled with anticoagulant (trisodium citrate [0.129 M, pH 6.0], enoxaparin [10 U/ml, Rhone-Poulenc Rorer, Collegeville, Pennsylvania], or CTI [32 μ g/ml, Fluka, Ronkonkoma, New York]).

After baseline blood samples were taken, subjects ingested 81 mg of aspirin daily for 5 days. A second phlebotomy was then performed. Subsequently, each subject underwent a 10-day washout period in which no antiplatelet agents were ingested. The protocol was then repeated with subjects ingesting 325 mg of aspirin daily, 250 mg of ticlopidine twice a day, or 250 mg of ticlopidine twice a day in combination with 325 mg of aspirin daily in each case. One subject did not complete the combination of aspirin and ticlopidine

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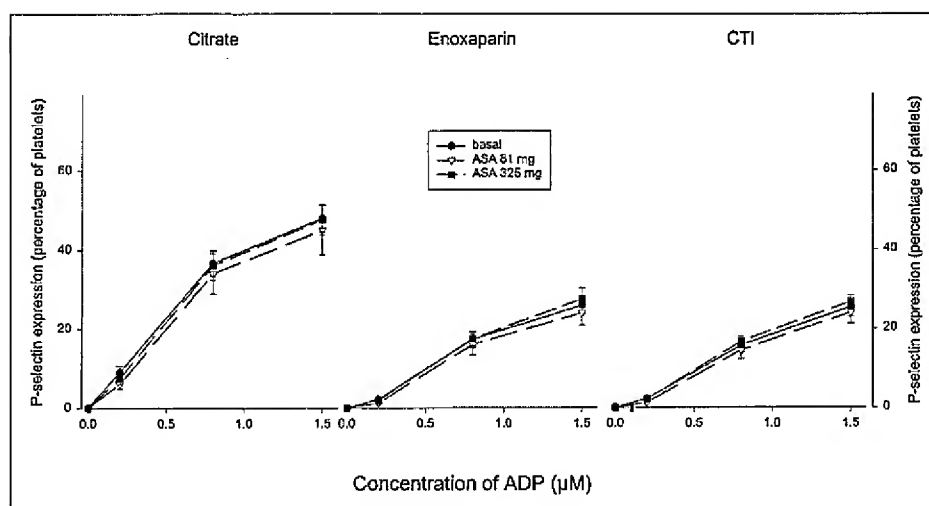


FIGURE 1. ADP-induced P-selectin expression in blood obtained from 9 subjects and anticoagulated with citrate, enoxaparin, and CTI (a specific inhibitor of factor XIIa). Degranulation of α granules was detected by the surface expression of P-selectin with a phycoerythrin conjugated anti-CD62 and determined with flow cytometry. After basal determination of platelet reactivity, subjects ingested 81 mg of aspirin (ASA) daily for 5 days. A 10-day washout period preceded repetition of the protocol with 325 mg of aspirin daily. Basal values represent the average determination of platelet reactivity before both regimens. The ingestion of aspirin did not inhibit ADP-induced P-selectin expression. Values are means \pm SEM.

regimen because of development of a rash after ingestion of ticlopidine alone.

Assays of platelet function: Flow cytometric assays were performed as previously described.¹² A 5- μ l aliquot of whole blood was incubated for 15 minutes in a reaction mixture that contained HEPES Tyrode buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM NaHCO₃, 0.36 mM NaH₂PO₄, 2 mM CaCl₂, 4 mM MgCl₂, and 5 mM dextrose, pH 7.4), a fluorescein isothiocyanate (FITC) conjugated antibody (HPI-1D) directed against the glycoprotein IIb/IIIa (activation independent), and a phycoerythrin (PE)-conjugated anti-CD62 IgG (Becton Dickinson, San Jose, California) directed against P-selectin. The FITC-HPI-1D was used to mark all platelets, and the anti-CD62 PE was used to mark platelets that had undergone α -granule degranulation. Assays were performed in triplicate with selected concentrations of ADP (0, 0.2, 0.8, 1.5 μ M). After a 15-minute incubation, platelets were fixed and red blood cells lysed with Optilyse-C (Immunotech, Westbrook, Maine). A control tube (containing FITC-HPI-1D and unfractionated immunoglobulin-G conjugated with PE) was included in each sample set to determine nonspecific antibody association with platelets. Association of antibodies with platelets was determined with the use of a fluorescence-activated cell sorter (Becton Dickinson).

Aggregometry was performed with platelet-rich plasma. Platelet-rich plasma was prepared conventionally by centrifugation (190 g, 15 minutes). Platelet-poor plasma was obtained by centrifugation of platelet-rich plasma (20,000 g, 3 minutes). Aggregation was performed in warmed (37°C) siliconized tubes, initiated with ADP (4 μ M) and collagen (0.19 mg/ml), and assayed in stirred platelet-rich plasma with the use of a PAP-4 aggregometer (BioData Corp, Horsham, Pennsylvania). Platelet-rich and platelet-

poor plasma were used to define 0% and 100% aggregation, respectively. We chose to initiate aggregation with 4 μ M ADP, because we have found that lower concentrations do not regularly initiate the second phase of aggregation. Collagen was used at a relatively high concentration to induce maximal aggregation.

Analysis of data: Values are means \pm SEM. Results from aggregation studies are presented with respect to baseline determinations. Thus, the results reported reflect the ratios of post-treatment to pretreatment values. Significant differences between pretreatment and post-treatment values for the same subject were identified with the use of a paired Student's *t* test. Comparisons between groups were performed with the use of analysis of variance.

RESULTS

Aspirin and platelet reactivity: The daily ingestion of aspirin (81 mg or 325 mg) for 5 days did not inhibit ADP-induced P-selectin expression (Figure 1). Similar results were obtained with blood anticoagulated with CTI, citrate, or enoxaparin. The ingestion of aspirin inhibited maximal aggregation induced by ADP in platelet-rich plasma anticoagulated with citrate, an effect variably observed by others (Figure 2). This effect was secondary to inhibition of the second phase of aggregation (Figure 3). In contrast, no effect of aspirin was seen on maximal aggregation induced by ADP in blood anticoagulated with CTI or with enoxaparin. Maximal aggregation induced by collagen was not affected by ingestion of aspirin. Collagen-induced aggregation in platelet-rich plasma anticoagulated with citrate displayed a trend toward inhibition of aggregation that was not apparent with CTI or enoxaparin (Figure 2). The limited inhibition of collagen-induced aggregation after ingestion of aspirin

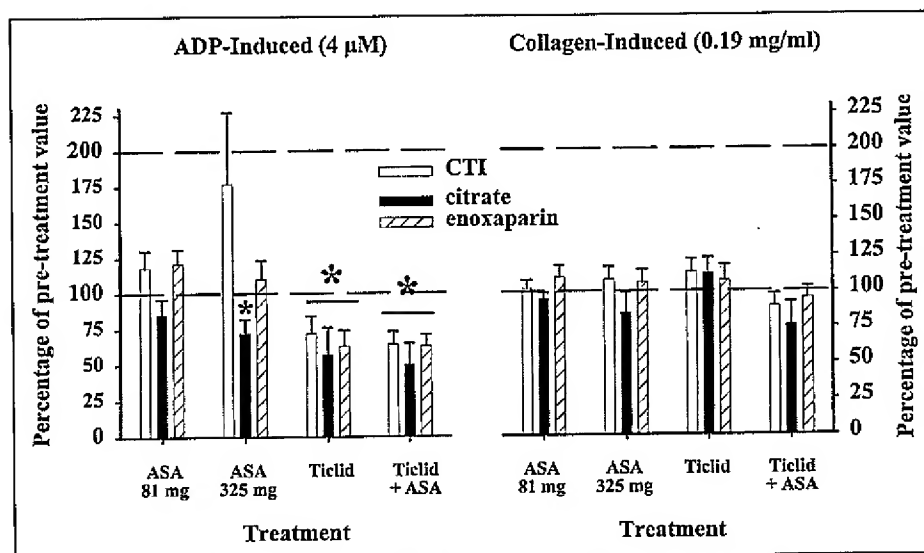


FIGURE 2. Maximal aggregation induced by 4 μ M ADP (right) and collagen (0.19 mg/ml, left) in platelet-rich plasma prepared from blood anticoagulated with citrate, enoxaparin, and CTI. Results are the percentage of pretreatment values from 9 subjects. After determination of pretreatment values, subjects ingested 81 mg of aspirin (ASA) daily for 5 days. A 10-day washout period preceded repetition of the protocol with 325 mg of aspirin daily, 250 mg of ticlopidine (Ticlid) twice daily, and ticlopidine plus aspirin 325 mg daily. Ingestion of 325 mg of aspirin daily inhibited maximal aggregation in platelet-rich plasma prepared from blood anticoagulated with citrate. Ingestion of ticlopidine and the combination of aspirin plus ticlopidine inhibited ADP-induced aggregation in blood treated with each anticoagulant. Inhibition of aggregation was similar after ticlopidine and aspirin plus ticlopidine. Values are means \pm SEM, * p < 0.05 when results were compared with those obtained during baseline studies. ADP-induced aggregation was inhibited (p < 0.05) after ingestion of aspirin and aspirin plus ticlopidine in each of the 3 anticoagulants.

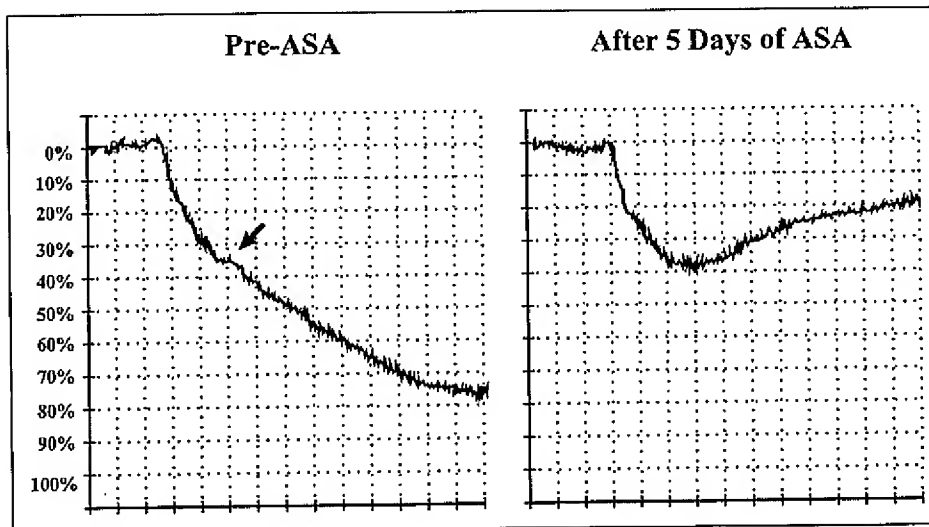


FIGURE 3. Aggregation as measured by aggregometry induced by 4 μ M ADP depicting representative results from 1 subject in platelet-rich plasma prepared from blood anticoagulated with citrate. Left, aggregation was induced by ADP before ingestion of 325 mg of aspirin daily for 5 days. First-phase and second-phase (arrow) aggregation are present. Right, aggregation was induced by ADP after ingestion of 325 mg of aspirin daily for 5 days. The second phase of aggregation does not occur, and disaggregation is apparent.

may be secondary to the concentration of collagen used in these studies.

Ticlopidine and platelet reactivity: The ingestion of ticlopidine inhibited ADP-induced expression of P-selectin (Figure 4). The inhibition was apparent with all concentrations of ADP tested and with blood anticoagulated with each of the 3 anticoagulants. On average, the inhibition of ADP-induced P-selectin expression was marked; however, substantial interindividual variability was apparent (Figure 5). Consistent with the known mechanism of action of ticlopidine,¹³ ingestion for 5 days inhibited ADP-induced aggregation but not collagen-induced aggregation (Figure 2).

Inhibition of ADP-induced aggregation was apparent with each of the anticoagulants. Similar to results with ADP-induced P-selectin expression, marked interindividual variability was apparent with ADP-induced aggregation.

Aspirin plus ticlopidine and platelet reactivity: The combination of aspirin and ticlopidine inhibited ADP-induced P-selectin expression (Figure 4). Platelet reactivity in platelet-rich plasma prepared from blood anticoagulated with enoxaparin and CTI was greater after ingestion of aspirin and ticlopidine than after ingestion of ticlopidine alone. Once again, substantial interindividual variability was apparent (Figure 5).

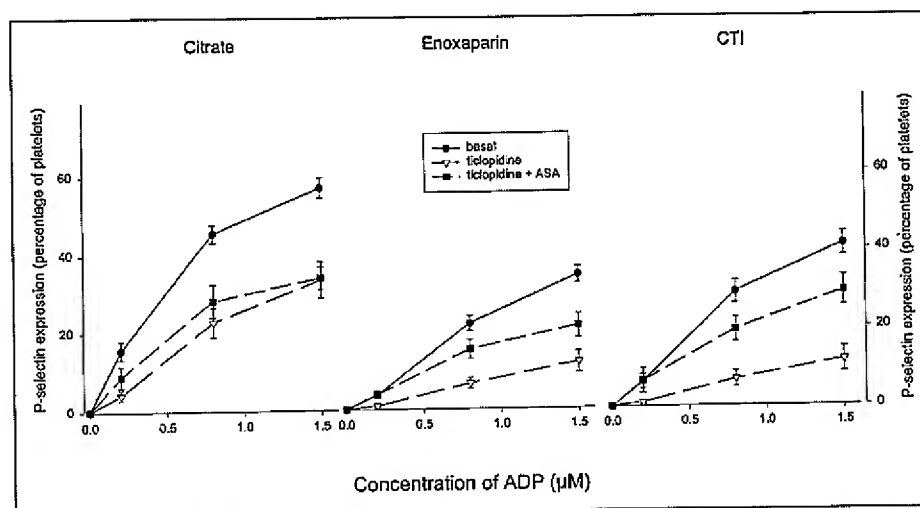


FIGURE 4. ADP-induced P-selectin expression in blood obtained from 9 subjects and anticoagulated with citrate, enoxaparin, and CTI (a specific inhibitor of factor XIIa). Degranulation of α granules was detected by the surface expression of P-selectin with a phycoerythrin-conjugated anti-CD62 and determined with flow cytometry. After basal determination of platelet reactivity, subjects ingested 250 mg of ticlopidine twice daily for 5 days. A 10-day washout period preceded repetition of the protocol with 250 mg of ticlopidine twice daily plus 325 mg of aspirin daily. Basal values represent the average determination of platelet reactivity before both regimens. The ingestion of ticlopidine and the ingestion of aspirin plus ticlopidine inhibited P-selectin expression in response to 0.8 and 1.5 μ M ADP in each anticoagulant ($p < 0.001$). The combination of aspirin and ticlopidine resulted in less inhibition of platelet reactivity than ticlopidine alone in blood anticoagulated with enoxaparin and CTI ($p < 0.05$). Values are means \pm SEM.

ADP-induced aggregation was inhibited by the combination of aspirin and ticlopidine (Figure 2). The inhibition was similar to that seen with ticlopidine alone. Aggregation induced by collagen was not reduced significantly by the combination of aspirin and ticlopidine (Figure 2). ADP-induced aggregation varied from subject to subject in a manner similar to that seen with respect to ADP-induced P-selectin expression.

DISCUSSION

Aspirin and platelet function: The involvement of platelets in thrombosis complicating plaque rupture and precipitating acute coronary syndromes has led to the development of treatment strategies designed to inhibit platelet function.^{2-5,14} Treatment with aspirin reduces the incidence of coronary events.^{2-5,14} One mechanism proposed is inhibition of platelet function. Roth and Majerus¹⁵ demonstrated that the exposure of platelets to aspirin inhibits cyclooxygenase and thus decreases production of thromboxane A_2 . Aspirin-induced acetylation of serine at position 530 inhibits cyclooxygenase activity through steric hindrance rather than through the modification of a catalytic function of cyclooxygenase.^{1,16,17}

Thromboxane A_2 , a platelet agonist, is released by activated platelets and leads to the recruitment of additional activated platelets. Based on our results, the effect of aspirin on platelet function appears to be mediated primarily through inhibition of formation of thromboxane A_2 . The ingestion of aspirin did not alter the activation process per se. That is, no change in the first phase of aggregation or in α -granule degranulation was seen after ingestion of aspirin for 5 days. Maximal aggregation induced by ADP was limited because of inhibition of the second phase of aggrega-

tion. Thus, ingestion of aspirin inhibited recruitment of additional activated platelets.

The inhibition of maximal aggregation induced by ADP in platelet-rich plasma prepared from blood anticoagulated with citrate was not apparent in platelet-rich plasma prepared from blood anticoagulated with CTI and enoxaparin. Thus, an inhibitory effect of aspirin was apparent only when platelet activation was assessed by aggregometry in response to ADP and in platelet-rich plasma prepared from blood anticoagulated with citrate. We have shown that the specific anticoagulant into which blood is drawn is a determinant of the observed reactivity of platelets.¹² Activation of platelets in response to agonists is increased when platelets are exposed to chelators of calcium, such as EDTA and citrate. Thus, demonstration of inhibitory effects of aspirin only after exposure of platelets to citrate suggests that the inhibition could be an artifact associated with exposure of platelets to citrate in vitro.

Ticlopidine and platelet activation: Ticlopidine is a prodrug that inhibits platelet function through inhibition of the ADP receptor.¹³ Maximal antiplatelet effects are apparent after 3 to 5 days of ingestion. We observed a marked inhibition of ADP-induced aggregation of platelets and ADP-induced P-selectin expression after ingestion of ticlopidine for 5 days. This effect was seen in blood anticoagulated with each of the anticoagulants studied.

The combination of aspirin plus ticlopidine inhibited ADP-induced aggregation and P-selectin expression. Consistent with our results with aspirin alone, the inhibition of platelet reactivity was not reduced further after ingestion of aspirin and ticlopidine compared with ticlopidine alone. Thus, no additive antiplatelet effect was seen with the combination after 5

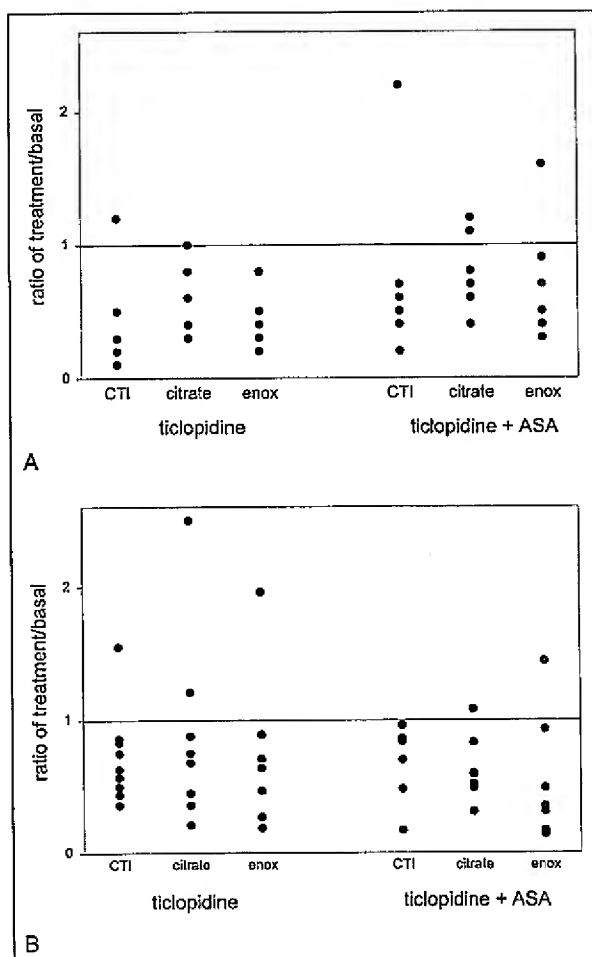


FIGURE 5. Expression of P-selectin in response to 0.8 μ M ADP (A) and aggregation induced by 4 μ M ADP (B) in blood obtained from 9 subjects and anticoagulated with citrate, enoxaparin, and CTI (a specific inhibitor of factor XIIa). Degranulation of α granules was detected by the surface expression of P-selectin with a phycoerythrin-conjugated anti-CD62 and determined with flow cytometry. After basal determination of platelet reactivity, subjects ingested 250 mg of ticlopidine twice daily for 5 days. A 10-day washout period preceded repetition of the protocol with 250 mg of ticlopidine twice daily plus 325 mg of aspirin daily. Values reflect the ratio of results after ingestion of the ticlopidine and ticlopidine plus aspirin divided by basal determination in each subject. Marked interindividual variability was apparent with both regimens in each anticoagulant.

days. These results are in contrast to those reported with platelets from patients treated with intracoronary stents.¹⁸ In this study, the combination of aspirin and ticlopidine led to a greater reduction in platelet reactivity than either aspirin alone or ticlopidine alone. Bossavy et al¹⁹ found that thrombosis initiated in vitro by tissue factor was inhibited to a greater extent by the combination of aspirin and ticlopidine than by either agent alone. Thus, additive antiplatelet effects of aspirin may be apparent after more prolonged exposure to aspirin, in subjects with atherosclerosis, and/or when thrombosis (the activation of both platelets and the coagulation cascade) is assessed.

Despite the efficacy of inhibition of platelet function that was seen when the group was analyzed as a

whole, marked interindividual variability was apparent after ingestion of ticlopidine alone and after the combination of ticlopidine plus aspirin. Thus, when results were analyzed in a given subject, the inhibition of platelet function ranged from minimal to marked. These results suggest that widespread availability of accurate and precise monitoring of platelet function should facilitate titration of therapy to optimize therapy for individual patients.

Clinical implications: Our results suggest that the combination of aspirin plus agents that inhibit primary activation events (such as ticlopidine, clopidogrel, and glycoprotein IIb to IIIa inhibitors) may be beneficial in patients at high risk for cardiac events. The marked interindividual variability in response to ingestion of ticlopidine suggests that widespread implementation of accurate and precise methods for assessment of platelet reactivity, such as the approach developed for the present study, will facilitate institution of optimal therapy tailored to each patient.

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Exhibit 33

Flow Cytometry Analysis of Intracellular VASP Phosphorylation for the Assessment of Activating and Inhibitory Signal Transduction Pathways in Human Platelets

Definition and Detection of Ticlopidine/Clopidogrel Effects

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Summary

Increased platelet adhesion or aggregation are key events in the pathogenesis of cardiovascular diseases. Exact determination of the platelet activation state is essential to recognize, prevent, and treat cardiovascular complications due to platelet dysfunction. Initial phases of platelet activation and inhibition are characterized by phosphorylation of specific intracellular proteins. However, methodological problems often prevent analysis of platelet protein phosphorylation under clinical conditions. A novel flow cytometry-based method using a phosphorylation-specific antibody was developed for fast and easy quantification of the phosphorylation state of a specific intracellular platelet protein. This method was used to analyze various platelet receptors and their intracellular signaling which may be impaired in genetic or acquired disorders or altered due to therapeutic interventions. In a first clinical application, the inhibitory effects of ticlopidine and clopidogrel on the platelet P2Y_{AC} ADP receptor were monitored.

Introduction

Platelet adhesion and activation are controlled via complex interactions between extracellular adhesive ligands, plasma membrane adhesion receptors, and intracellular cytoskeletal proteins. This interplay is coordinated by intracellular signaling molecules that regulate the activation state of platelets [reviewed in (1, 2)]. Increased platelet adhesion or platelet aggregation are crucial pathological events in arterial cardiovascular diseases. Platelet activation can occur under pathological blood flow conditions in areas of disrupted endothelial monolayer, as well as by a variety of agents released from activated and/or injured cardiovascular cells including platelets. Release of adenosine diphosphate (ADP) has been recognized as a major factor in development of arterial thrombosis (3, 4). This is also supported by the demonstration

that the thienopyridine derivatives ticlopidine and clopidogrel which selectively block ADP-induced platelet activation are very effective for treatment of vascular diseases associated with increased platelet activation (5–7). Exact determination of the platelet activation state is therefore a desirable clinical parameter to recognize and prevent cardiovascular complications due to platelet dysfunction. Surface expression of P-selectin (CD62P) or formation of microparticles are widely used markers of platelet activation (8–10). These changes on the platelet surface, and formation of aggregates are easily detectable by means of flow cytometry, however, they appear in the later phases of platelet activation and also do not indicate all activation responses. Early changes in platelet activation involve phosphorylation of intracellular platelet proteins such as platelet p42 mitogen-activated protein kinase (MAPK), pleckstrin, as well as other intracellular signaling molecules (11, 12). Furthermore, inhibition of platelet activation (e.g. by vasodilators or endothelium-derived factors) induces phosphorylation of various intracellular signaling molecules without affecting expression of platelet surface proteins [reviewed in (13)]. The vasodilator-stimulated phosphoprotein (VASP), a highly concentrated, cytoskeleton- and integrin-associated platelet protein, is such a marker protein for platelet inhibition, since its phosphorylation correlates very closely with inhibition of binding of soluble fibrinogen to platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) and inhibition of platelet aggregation (14). VASP has three different phosphorylation sites that are used by both cGMP- and cAMP-dependent protein kinases, however each kinase has a different preference for the individual phosphorylation sites (15). Recently, a phosphorylation-specific monoclonal antibody (16C2) was developed that recognizes phosphorylation at serine 239, a site preferred by cGMP-dependent protein kinase, but used by both cAMP- and cGMP-dependent protein kinases *in vitro* and in intact cells (16).

Here, we report the development of a new flow cytometry-based method to access detection of intracellular platelet protein phosphorylation using a phosphorylation-specific monoclonal antibody. This technique was used to analyze intracellular inhibitory and stimulatory pathways in human platelets, and to characterize several platelet-activating receptors. In a first clinical application, the *in vivo* effects of thienopyridine derivatives (ticlopidine/clopidogrel) on the platelet P2Y_{AC} ADP receptor were detected and monitored by *ex vivo* flow cytometry experiments.

Materials and Methods

Materials

Antibody 16C2 directed against serine 239-phosphorylated VASP and the polyclonal M4 antibody have been described (16, 17). The cyclic nucleotide

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Abbreviations: ADP: adenosine 5'-diphosphate; cAMP: cyclic adenosine-3',5'-monophosphate; cGMP: cyclic guanosine-3',5'-monophosphate; HUVECs: human umbilical vein endothelial cells; MAPK: mitogen-activated protein kinase; PG-E₁: prostaglandin E₁; PRP: platelet-rich plasma; SNP: sodium nitroprusside; VASP: vasodilator-stimulated phosphoprotein

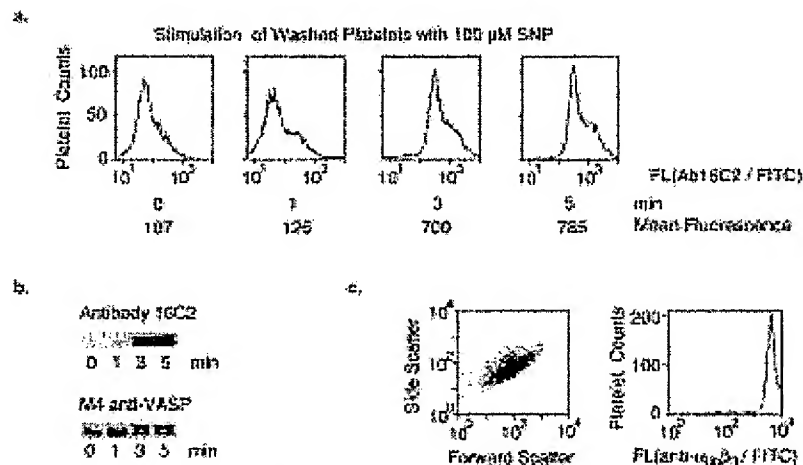


Fig. 1 Sodium nitroprusside (SNP)-induced VASP phosphorylation in washed human platelets detected by flow cytometry (a) or Western blotting (b). Washed human platelets were incubated with 100 μ M SNP; at the time points indicated, aliquots of the platelet suspension were removed and stopped for Western blotting, or fixed with formaldehyde for flow cytometry analysis. Fixed platelets were then permeabilized and stained with phosphorylation-specific antibody 16C2 as described in Methods. Panel a) shows original flow cytometry histograms of antibody 16C2 binding to serine 239-phosphorylated platelet VASP. Analyzed time points and mean fluorescence intensities are indicated. Panel b) shows an autoradiogram of Western blot analysis using serine 239 phosphorylation-specific antibody 16C2 and a polyclonal VASP antiserum (M4) that recognizes both dephosphorylated and phosphorylated VASP. Panel c) indicates the analyzed region in a forward/side scatter dot blot of platelet flow cytometry analysis and the fluorescence signal of binding of a platelet-specific antibody against integrin $\alpha_{IIb}\beta_3$.

analogs 8-pCPT-cGMP and Sp-5,6-DCl-cBIMPS were obtained from BioLog (Bremen, Germany). Ticlopidine (Tiklyd™) and clopidogrel (Plavix™) were provided from Sanofi Winthrop (Munich, Germany).

Isolation, Stimulation, Sample Preparation, and Flow Cytometry Analysis of Platelet-Rich Plasma or Washed Human Platelets

Platelet-rich plasma (PRP) or washed human platelets were isolated and stimulated with vasodilators as described previously (17, 18). At various time points, aliquots of the cell suspension were removed, and reactions were stopped for Western blot analysis by addition of 30% final concentration of SDS-stop solution as described previously (17). For flow cytometry, 3% (final concentration) of methanol-free formaldehyde was added, and samples were allowed to fix for 5 min at room temperature. Concentrations between 2% and 5% of formaldehyde were tested and showed identical results of platelet VASP phosphorylation in flow cytometry analysis. Then, platelets were pelleted for 10 s at 8000 \times g, resuspended in 0.2% Triton X-100 in phosphate-buffered saline (PBS), and permeabilized for 10 min at room temperature. Platelets were pelleted for 1 min at 2700 \times g and resuspended in PBS. Antibody 16C2 (anti-phospho-VASP) was added in a final concentration of 2.4 μ g/ml, and incubated for 30 min at room temperature. Then, the platelet suspension was centrifuged for 1 min at 2700 \times g, platelet pellets were resuspended in PBS with 25 μ g/ml fluorescein isothiocyanate (FITC)-conjugated, affinity-purified goat anti-mouse IgG (Sigma, St. Louis, MO), and incubated for 20 min at 4° C in the dark. Platelets were washed one more time with PBS, and analyzed at low flow rate on a Becton Dickinson FACSCalibur. The instrument settings used were: forward scatter: E00, side scatter: 337 V, fluorescence channel 1: 600 V. All three detectors were set to logarithmic amplification. The platelet population was identified on its forward and side scatter distribution, and 15,000 platelet events were analyzed for mean fluorescence using CELLQuest software, version 3.1f.

Stimulation, Sample Preparation, and Flow Cytometry Analysis of Whole Blood

Citrate-anticoagulated blood was incubated with vasodilators at 37° C. At various time points, aliquots were removed, and reactions were stopped by addition of 3% final concentration of methanol-free formaldehyde and allowed to

fix for 10 min at room temperature. Then, samples were centrifuged for 20 s at 2900 \times g in a microcentrifuge, and PRP-like supernatant was transferred to a new microfuge tube. Platelets were pelleted for 10 s at 8000 \times g, resuspended in 0.2% Triton X-100 in PBS, and permeabilized for 10 min at room temperature. Staining with antibodies and flow cytometry analysis of platelets were performed as described above for platelet-rich plasma and washed human platelets.

Western Blotting and Quantification

Western blotting onto nitrocellulose was performed as described previously (17) using 6% powdered milk in phosphate-buffered saline with 0.05% Tween-20 (PBS/Tween) as a blocking solution and 3% powdered milk in PBS/Tween for antibody incubation. Antibody 16C2 was used in a concentration of 0.5 μ g/ml. Immunoreactivity was determined using affinity-isolated, peroxidase-conjugated goat anti-mouse IgG (1:3000) (BioRad, Hercules, CA) and the ECL chemiluminescence reaction (Amersham Corp., Arlington Heights, IL). Intensity of these Western blot bands was quantified by densitometry on a Macintosh computer using NIH Image software, version 1.61. All labeled bands were analyzed within the linear range for the chemiluminescence reaction.

Incubation of Whole Blood with Human Umbilical Vein Endothelial Cells

A monolayer of cultured human umbilical vein endothelial cells (HUVECs) was coincubated with citrate-anticoagulated whole blood as described previously (19). At various time points, aliquots of whole blood were removed and processed for flow cytometry analysis as described above.

Volunteers

Thienopyridine derivatives effects were studied in platelets obtained from healthy volunteers (age range 23-49 years) who received a 7-day medication of either ticlopidine (500 mg/day) or clopidogrel (75 mg/day). Blood samples were taken before, on several days during, and up to 4 weeks after the end of treatment. Preparation and stimulation of platelet-rich plasma (PRP), and analysis of platelet VASP phosphorylation were performed as described above. All investigations reported here were approved by the ethics committee of our university and performed according to the guidelines of the declaration of Helsinki. Informed consent was obtained from all volunteers.

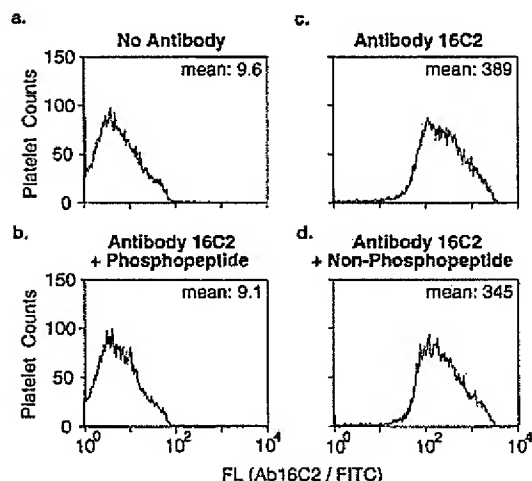


Fig. 2 Specificity of flow cytometry analysis of serine 239-phosphorylated VASP in human platelets using antibody 16C2. Washed platelets were incubated for 3 min with 10 μ M PG- E_1 to stimulate VASP phosphorylation. Flow cytometry analysis was performed without addition of a primary antibody (a), with addition of antibody 16C2 which had been preincubated with a 200-fold molar excess of a VASP peptide containing phosphorylated serine 239 (b), with addition of antibody 16C2 (c), or with addition of antibody 16C2 which had been preincubated with a 200-fold molar excess of an identical VASP peptide with non-phosphorylated serine 239 (d). Mean values of fluorescence are indicated, and a representative experiment is shown

Results

Flow Cytometry Analysis of Intracellular Platelet Protein Phosphorylation

The phosphorylation-specific antibody 16C2 is an established tool to analyze vasodilator-stimulated phosphoprotein (VASP) phosphorylation in cardiovascular cells by standard Western blot technique (16). However, exact quantification of phosphorylated VASP by this technique is time consuming, and cannot be performed with whole blood. Therefore, we investigated, whether flow cytometry can be used for fast and easy quantification of specific platelet VASP phosphorylation. Washed human platelets were incubated with 100 μ M sodium nitroprusside (SNP), and at certain time points aliquots of the platelet suspension were removed, immediately fixed, permeabilized, and stained with antibody 16C2 as described in Methods. Analysis by flow cytometry detected a time-dependent increase in fluorescence signal corresponding to increased VASP phosphorylation (Fig. 1a). The increased flow cytometry fluorescence signal correlated closely to increased VASP phosphorylation detected by parallel Western blot analysis (Figs. 1b and 3). The amount of VASP in the samples was not affected by this treatment as determined by flow cytometry or Western blot technique using a polyclonal anti-VASP antibody (antibody M4) that recognized both phospho- and dephospho-VASP (Fig. 1b, flow cytometry data not shown). Parallel staining with a platelet-specific antibody against integrin $\alpha_{IIb}\beta_3$ ensured platelet identity of the analyzed events (Fig. 1c).

Specificity of Flow Cytometry Analysis

To demonstrate specificity of flow cytometry analysis of intracellular VASP phosphorylation, antibody 16C2 was preincubated with

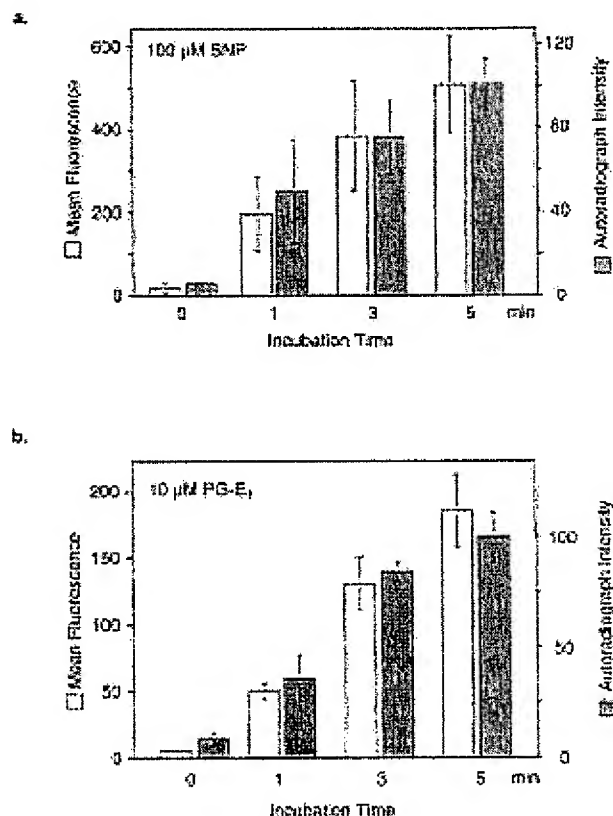


Fig. 3 Comparison of vasodilator-stimulated VASP phosphorylation at serine 239 in washed human platelets analyzed by flow cytometry or Western blot technique. Washed human platelets were incubated with 100 μ M SNP (a) or 10 μ M PG- E_1 (b), at the time points indicated aliquots of the platelet suspension were removed and analyzed by flow cytometry or Western blotting in parallel. The histograms demonstrate the mean fluorescence of 16C2 binding analyzed by flow cytometry (open bars), or Western blot detection of VASP phosphorylation using antibody 16C2 and image analysis of the autoradiograms (filled bars). Data represent means \pm SEM of 4 independent experiments

peptides representing the recognition sequence of antibody 16C2 (VASP amino acids 232 to 246) with phosphorylated or non-phosphorylated serine 239, and then used for flow cytometry analysis. The serine 239-phosphorylated peptide blocked antibody 16C2 binding to phosphorylated platelet VASP completely (compare Figs. 2a and 2b), while the non-phosphorylated control peptide had no effect on antibody 16C2 binding (compare Figs. 2c and 2d). Furthermore, staining of unstimulated and prostaglandin-stimulated platelets with unspecific antibodies of the same isotype as antibody 16C2 led to fluorescence intensities in the magnitude of the background signal with secondary antibody alone (data not shown). The analysis of specificity was performed in washed platelets as well as in whole blood.

Comparison of Flow Cytometry and Western Blot Analysis

To confirm that results of flow cytometry analysis of VASP phosphorylation are equal to data obtained by an established method like Western blotting, we stimulated VASP phosphorylation in washed human platelets with various vasodilators, removed identical samples, and analyzed them in parallel by flow cytometry or Western blotting. Autoradiograms were quantified by image analysis. Comparison of

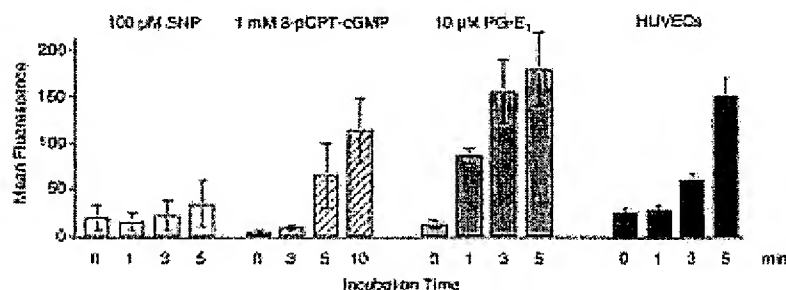


Fig. 4 Time course of platelet VASP phosphorylation in whole blood after stimulation with sodium nitroprusside, the cGMP analog 8-pCPT-cGMP, prostaglandin E₁, or after coincubation with human umbilical vein endothelial cells (HUVECs) analyzed by flow cytometry. Citrate-anticoagulated whole blood was incubated with 100 μM SNP (open bars), 1 mM 8-pCPT-cGMP (hatched bars), 10 μM PG-E₁ (grey bars), or coincubated with a monolayer of HUVECs (dark bars). At the time points indicated aliquots of the platelet suspension were removed and analyzed for serine 239 VASP phosphorylation by flow cytometry as described in Methods. Histograms show mean fluorescence of antibody 16C2 binding. Data represent means ± SEM of 3 independent experiments

flow cytometry and Western blot analysis results demonstrated a close correlation between both methods after stimulation of platelets with 100 μM SNP (Fig. 3a) or 10 μM prostaglandin E₁ (PG-E₁) (Fig. 3b). Close correlation was also found after stimulation of platelets with lower concentrations of these vasodilators (1 μM SNP or 10 nM PG-E₁) or stimulation with cell membrane-permeable cGMP or cAMP analogs (1 mM 8-pCPT-cGMP or 1 mM Sp-5,6-DCI-cBIMPS, respectively) (data not shown).

Flow Cytometry Analysis of Intracellular Platelet VASP Phosphorylation in Whole Blood

In contrast to most classical methods for detection of protein phosphorylation, flow cytometry analysis of surface (8-10) and intracellular proteins can be easily performed with whole blood samples. We com-

pared the ability of various vasodilators to induce platelet VASP phosphorylation in whole blood. Stimulation of whole blood with 100 μM SNP induced very poor platelet VASP phosphorylation (Fig. 4). This weak effect of SNP (in contrast to the strong effects observed with washed platelets) might be due to the presence of free hemoglobin in our whole blood assay system, since incubation with 1 mM of the cGMP analog 8-pCPT-cGMP induced strong VASP phosphorylation (Fig. 4) that was comparable to the fluorescence signal obtained in washed platelets after stimulation with this analog (data not shown). Furthermore, incubation of whole blood with 10 μM PG-E₁ induced VASP phosphorylation similar to that in washed platelets (Figs. 3 and 4). Indeed, 0.53 ± 0.04 g/l free hemoglobin (mean ± SEM of 4 independent measurements) was detected in the PRP fraction of a whole blood sample. Free hemoglobin as well as hemoglobin within washed erythrocytes have been shown to inhibit nitric oxide (NO)

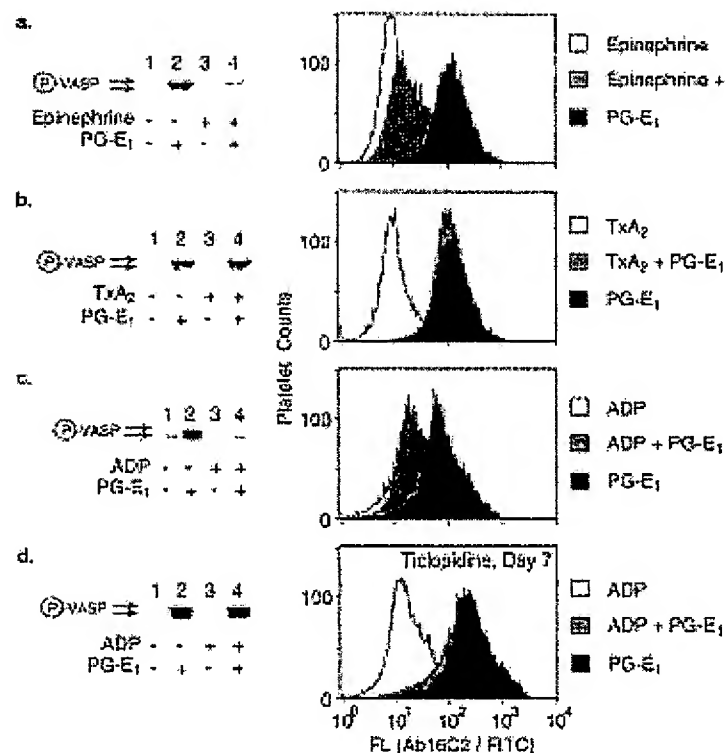


Fig. 5 Analysis of receptor-regulated intracellular signaling pathways in platelets by Western blotting or flow cytometry. Platelet-rich plasma (PRP) was incubated for 3 min with 3 μM PG-E₁ without or with 55 μM epinephrine, 1 μM of the thromboxane analog U46619 (TxA₂), or 20 μM ADP as indicated. Analysis of platelet VASP phosphorylation using antibody 16C2 was performed as described in Methods. A representative Western blot (left panel) and a representative original flow cytometry histogram overlay (right panel) is shown for each substance (panel a to c). Panel d shows effects of a 7-day ticlopidine treatment (500 mg/day) on ADP-induced inhibition of PG-E₁-stimulated platelet VASP phosphorylation

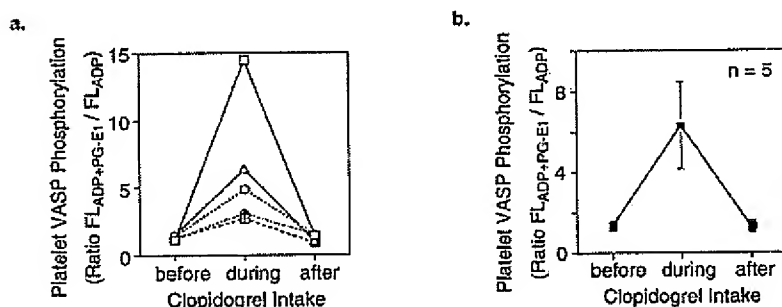


Fig. 6 Effects of clopidogrel on ADP-induced inhibition of PG-E₁-stimulated platelet VASP phosphorylation. PRP of 5 volunteers was analyzed before, at the end of a 7-day clopidogrel intake, and 4 weeks after clopidogrel treatment was stopped. PRP was incubated for 3 min with 5 μ M ADP alone or both 30 nM PG-E₁ and 5 μ M ADP, and analyzed for platelet VASP phosphorylation by flow cytometry. Fluorescence intensities are corrected for non-specific binding of secondary antibody. Mean fluorescence of platelets stimulated with ADP alone was only slightly above background staining. Fluorescence data shown in this figure indicate the extent of VASP serine 239 phosphorylation evoked by the combination of ADP and PG-E₁ relative to that observed with ADP alone (FL_{ADP+PG-E1}/FL_{ADP}). A factor of 1.0 or below indicates complete inhibition of PG-E₁-stimulated VASP phosphorylation by ADP, a factor >1.0 indicates detectable PG-E₁-stimulated VASP phosphorylation despite the presence of ADP. In panel a data are shown for each individual. Panel b shows mean \pm SEM of all 5 volunteers.

effects on human platelets (20–22). To test whether factors released from human endothelial cells can induce detectable VASP phosphorylation in this system, whole blood was incubated with a nearly confluent monolayer of human umbilical vein endothelial cells (HUVECs). At certain time points, aliquots of the blood were removed and analyzed by flow cytometry. Factors released from HUVECs induced rapid and strong phosphorylation of platelet VASP *in vitro* (Fig. 4). Flow cytometry analysis of platelet protein phosphorylation requires very little blood volumes. Detection of PG-E₁-stimulated platelet VASP phosphorylation in as little as 30 μ l whole blood obtained from a mouse was successful (data not shown).

Flow Cytometry Analysis of Receptor-Regulated Intracellular Signaling Pathways

Flow cytometry of intracellular protein phosphorylation is a useful tool to assess and characterize platelet receptors and their intracellular signaling mechanisms. Several receptor-dependent platelet activators are known, e.g. ADP, thrombin, epinephrine, or thromboxane A₂ (TxA₂). To test effects of these activators on platelet VASP phosphorylation, platelet-rich plasma was incubated with buffer, 20 μ M ADP, 55 μ M epinephrine or 1 μ M of the TxA₂ analog U46619 in the presence or absence of 3 μ M PG-E₁. Incubation of PRP with epinephrine, U46619 or ADP alone for 3 min did not induce VASP phosphorylation (Fig. 5, lane 3). Incubation with PG-E₁ induced strong VASP phosphorylation in platelets at serine 239 as demonstrated by Western blotting (Fig. 5, lane 2). However, co-stimulation of platelets with PG-E₁ and epinephrine or ADP almost completely inhibited PG-E₁-induced VASP phosphorylation (Figs. 5a and 5c, lane 4, and flow cytometry histograms), an effect also observed with 1 U/ml thrombin in washed platelets (data not shown). In contrast, co-incubation of PG-E₁ and U46619 had no inhibitory effect on PG-E₁-stimulated VASP phosphorylation (Fig. 5b, lane 4, and flow cytometry histogram).

In Vivo Monitoring of Platelet Inhibition by Thienopyridines

Thienopyridines (ticlopidine and clopidogrel) are potent inhibitors of ADP-induced platelet activation, resulting in nearly complete inhibition of ADP-stimulated platelet aggregation (5, 7). As previously shown, thienopyridines inhibit a G_i protein-coupled P2Y_{AC} ADP recep-

tor in platelets, which is also responsible for the inhibitory effect of ADP on platelet adenylyl cyclase (23). *In vivo* treatment with ticlopidine should therefore attenuate the ADP-induced inhibition of cAMP-stimulated VASP phosphorylation.

Two volunteers were treated for 7 days with ticlopidine, blood samples were taken before (day 0), at several time points during (days 1 to 7) and for up to 3 weeks after ticlopidine intake. Blood of a non-treated control person was analyzed at the same time points. PRP was isolated from these samples, stimulated with ADP, PG-E₁, or both ADP and PG-E₁ for 3 min and then analyzed for VASP phosphorylation by flow cytometry. After 4 days of ticlopidine intake, ADP-regulated inhibition of PG-E₁-induced VASP phosphorylation was suppressed,

Table 1 Time course of ticlopidine effects on platelet VASP phosphorylation

	control	volunteer 1	volunteer 2
day 0	1.0	1.0	0.9
day 2	1.1		
day 4	0.8		
day 7	1.2		
day 10	1.1	4.8	2.2
day 14	1.0	3.9	1.5
day 16	n.d.*	1.0	1.4
day 25	0.9	1.2	0.9

* n.d.: not determined

Flow cytometry data of platelet VASP phosphorylation in PRP of a control person (no medication) and two volunteers at several time points before, during, and after ticlopidine treatment (500 mg/day) are shown. The shaded area indicates the period of ticlopidine medication (day 1 to day 7). At each day of analysis PRP was incubated with 5 μ M ADP alone or both 30 nM PG-E₁ and 5 μ M ADP for 3 minutes, and analyzed for platelet VASP phosphorylation by flow cytometry. Fluorescence intensities were corrected for non-specific binding of secondary antibody. Fluorescence data shown in this table indicate the extent of VASP serine 239 phosphorylation evoked by the combination of ADP and PG-E₁ relative to that observed with ADP alone (FL_{ADP+PG-E1}/FL_{ADP}). A factor of 1.0 or below indicates complete inhibition of PG-E₁-stimulated VASP phosphorylation by ADP, a factor > 1.0 indicates detectable PG-E₁-stimulated VASP phosphorylation despite the presence of ADP.

and a marked increase in VASP phosphorylation was detectable after stimulation with PG-E₁ and ADP compared to the analysis prior to ticlopidine intake (Table 1. See also the corresponding Western blot analysis in Figs. 5c and d). The untreated control person showed complete inhibition of PG-E₁-induced VASP phosphorylation at all analyzed time points (Table 1). The ticlopidine effect reached its maximum between day 4 and day 10, depending on the individual volunteer, and was detectable for up to 11 days after ticlopidine intake was stopped (Table 1). All ADP effects were normalized again 18 days after ticlopidine treatment was ended (Table 1).

In a follow-up study with 5 volunteers, ADP-induced suppression of VASP phosphorylation was analyzed before, at the end of a 7-day clopidogrel treatment, and 4 weeks after clopidogrel intake was stopped. Fig. 6 demonstrates complete inhibition of PG-E₁-stimulated VASP phosphorylation by ADP before and 4 weeks after clopidogrel intake was discontinued, however, clopidogrel treatment blocked this ADP inhibitory effect and increased VASP phosphorylation significantly ($p = 0.04$, paired t-test) (Figs. 6a and b). The individual volunteers responded differently to clopidogrel (Fig. 6a), an effect that correlated with comparable differences in the degree of inhibition of ADP-induced platelet aggregation (data not shown), and might reflect differences in the responsiveness to thienopyridines.

Discussion

The results of this study allow three major conclusions:

1. Flow cytometry is capable of detecting and quantifying specific intracellular protein phosphorylation in human platelets, as demonstrated by the analysis of VASP phosphorylation.
2. Flow cytometry measurement of VASP phosphorylation allows the functional characterization of several human platelet receptors (e.g. ADP, thrombin, prostaglandin receptors) and intracellular signaling pathways (NO-, cGMP-, and cAMP-mediated pathways).
3. Flow cytometry analysis of VASP phosphorylation in human platelets detects an inhibitory effect of ticlopidine and clopidogrel on ADP-regulated VASP phosphorylation which is mediated by the platelet P2Y_{AC} ADP receptor.

Previously, flow cytometry analysis of platelet function has been primarily used to detect activation markers on the platelet surface (e.g. P-Selectin CD62P), changes in the activation state of surface receptors (e.g. integrin $\alpha_{IIb}\beta_3$), and formation of platelet microparticles or aggregates [reviewed in (10)]. However, these parameters indicate only final steps of platelet activation. For the early phases of platelet activation by platelet agonists, classical biochemical methods have demonstrated a dramatic increase in phosphorylation of several signaling molecules such as p42 MAPK (at threonine 202 and tyrosine 204), several non-receptor-type protein tyrosine kinases like pp60src and pp72syk and the focal adhesion kinase pp125FAK (11, 24-27). In a first approach we successfully used antibody 12D4 which is directed against di-phosphorylated p42/p44 MAPK for detection of MAPK activation in thrombin-stimulated platelets using flow cytometry (data not shown). In the future, it should be possible to extend these studies to other proteins phosphorylated during platelet activation. Molecules of interest include protein tyrosine kinases and their substrates, myosin light chains, the protein kinase C substrate pleckstrin, and cytoplasmic domains of integrin $\alpha_{IIb}\beta_3$ (12, 24-32).

However, understanding of platelet function not only requires the analysis of activators and activation cascades but also the measurement of inhibitory pathways. Potent platelet inhibitors like cyclic nucleotide-elevating vasodilators and/or endothelial factors (i.e. NO and prosta-

cyclin) stimulate the phosphorylation of certain intracellular proteins like VASP, rap I-b, MLCK and other signaling molecules [reviewed in (13)]. The focal adhesion-associated protein VASP is the only common substrate of both cGMP- and cAMP-dependent protein kinases which are activated in response to cGMP- and cAMP-elevating platelet inhibitors, respectively (13-19). VASP phosphorylation closely correlates with inhibition of platelet activation and in particular with inhibition of soluble fibrinogen binding to its receptor integrin $\alpha_{IIb}\beta_3$ (1, 14). Therefore, VASP phosphorylation has been found to be a useful marker for platelet inhibition.

However, the rapid time course of VASP phosphorylation/dephosphorylation in intact human platelets (17) and the limitations of conventional biochemical techniques so far permitted the analysis of VASP phosphorylation only in washed platelets or PRP. Here, flow cytometry analysis with the newly-developed monoclonal antibody 16C2 demonstrated that this technique can be used to quantify serine 239-specific VASP phosphorylation in washed human platelets, and platelets of PRP and whole blood (Figs. 1-4). Whenever a direct comparison was possible, the extent of VASP phosphorylation measured by flow cytometry closely correlated with that observed by the conventional Western blot technique (Figs. 1 and 3). The possibility of measuring the state of VASP phosphorylation in whole blood by flow cytometry could be of considerable clinical relevance. Direct fixation of whole blood immediately after obtaining the blood sample, without any further manipulation, followed by flow cytometry could measure the state of *in vivo* VASP phosphorylation at the time point and site at which the blood sample was obtained. Since VASP serine 239 is phosphorylated in response to both cAMP- and cGMP-elevating agents such as NO and prostacyclin, VASP phosphorylation analyzed by flow cytometry may therefore be useful to monitor *in vivo* endothelial function/dysfunction and the *in vivo* effects of NO-, prostaglandin- and phosphodiesterase-based platelet inhibitors and vasodilators. Recently, it was shown that acetylcholine-stimulated NO release from endothelial cells inhibited platelet aggregation, and that this effect was attenuated in patients with atherosclerosis and endothelial dysfunction (33). Our experiments with cultured human endothelial cells (Fig. 4) indicate that the technique described is suitable to detect endothelial cell-platelet interactions.

The measurement of VASP serine 239 phosphorylation by flow cytometry can also be used for functional analysis of platelet activators and their receptors that negatively influence cAMP-regulated signal transduction pathways. Both Western blot and flow cytometry analysis demonstrated that epinephrine, ADP, and thrombin (but not the thromboxane analog U46619) strongly inhibited PG-E₁-stimulation of VASP serine 239 phosphorylation (Fig. 5, thrombin data not shown). These data agree with published information that some platelet activators such as thrombin, epinephrine and ADP activate G_i protein-coupled receptors that induce the release of G α_i subunits and inhibition of adenylyl cyclase (34). Our data demonstrate that these G_i-coupled receptors can be analyzed by flow cytometry which should be helpful for the rapid analysis and screening of diseases and conditions in which receptor defects or impairment are suspected. Single case reports of changes in phosphorylation or intracellular signaling have been described. In two independent patients with inherited bleeding disorders, a defective interaction between ADP and its receptors on platelets was found (35, 36). In these platelets, fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ was decreased, and ADP was unable to lower cAMP levels, an effect easily detectable by the determination of platelet VASP phosphorylation. A variety of other genetic or acquired defects in human platelets which may contribute to enhanced risk of thrombotic or hemorrhagic disorders have been identified, i.e. an inherited defect in platelet responses to

nitric oxide in a family with history of arterial thrombosis, and deficiencies in cGMP-regulated platelet signaling in patients with chronic myelocytic leukemia (18, 37). Platelet signal transduction defects with G α subunit dysfunction and diminished G α in a patient with abnormal platelet responses have been reported (38). Defective signal transduction through the thromboxane A₂ receptor in a patient with a mild bleeding disorder resulted in deficiency of the inositol 1,4,5-trisphosphate formation, probably due to a defect in phospholipase C activation (39).

The flow cytometry method for detection of intracellular platelet protein phosphorylation will allow rapid and easy screening for such deficiencies in receptor/enzyme activities. In a first clinical application, we used this method for the analysis of platelet inhibitors thought to function as ADP receptor antagonists. The thienopyridines ticlopidine and clopidogrel selectively impair ADP-stimulated platelet aggregation but their precise mechanism of action is not well understood (5, 7). Recently, our group (23) and others (40-43) identified and characterized three distinct human platelet ADP receptors. Treatment of human volunteers with ticlopidine did not affect the ADP receptors coupled to rapid calcium influx (P2X₁ ADP receptor) and calcium mobilization (P2Y₁ ADP receptor) (23), an effect also observed in platelets from clopidogrel-treated rats (44) or human platelets from volunteers treated with clopidogrel (45). Our present data, obtained by both Western blot and flow cytometry analysis, demonstrate that treatment of human volunteers with either ticlopidine or clopidogrel strongly attenuated the inhibitory effect of ADP (but not that of epinephrine, data not shown) on prostaglandin E₁-stimulated, cAMP-mediated VASP serine 239 phosphorylation (Figs. 5 and 6, and Table 1). These results also demonstrate that the G_i protein-coupled human platelet P2Y_{AC} ADP receptor is the primary *in vivo* target of the thienopyridine-based platelet inhibitors ticlopidine and clopidogrel. This P2Y_{AC} ADP receptor, which has not yet been cloned, is impaired by these platelet inhibitors with remarkable selectivity since other ADP receptors and other G_i protein-coupled receptors were not affected. Our data with respect to time course, extent, and wash-out phase also indicate that flow cytometry analysis of VASP phosphorylation is a useful method to monitor the *in vivo* effects of ticlopidine and clopidogrel which are known to be essentially irreversible platelet inhibitors requiring *in vivo* activation/metabolism (5, 7).

In conclusion, flow cytometry analysis of platelet VASP phosphorylation has been demonstrated here as a new parameter for the analysis of platelet function which should be useful in the future for both basic and clinical investigations. Applications include the analysis of platelet inhibitors with respect to their mechanisms of action and monitoring of their therapeutic efficacy. Other applications include the analysis of genetic and acquired defects in platelet receptors and signaling. Also, the possibility of analysing mouse and rat platelets (Eigenthaler M, unpublished data) should be of considerable interest considering the important role of genetic rodent models for cardiovascular research. Clearly, flow cytometry measurement of protein phosphorylation is not limited to the analysis of VASP phosphorylation as demonstrated here. The increasing availability of phosphorylation-specific antibodies in combination with the method developed here will broaden the possibilities of flow cytometry analysis in clinical diagnosis and research.

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Exhibit 34

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Specific Impairment of Human Platelet P2Y_{AC} ADP Receptor–Mediated Signaling by the Antiplatelet Drug Clopidogrel

J. Geiger, J. Brich, P. Hönig-Liedl, M. Eigenthaler, P. Schanzenbächer, J.M. Herbert, U. Walter

Abstract—Clopidogrel is an effective new antiplatelet agent useful for the treatment of ischemic cerebrovascular, cardiac, and peripheral arterial disease. However, the mechanism of clopidogrel action is not well understood, although it is known to inhibit ADP-evoked platelet aggregation. In the current study, the effect of clopidogrel on recently identified human platelet ADP receptors and their signaling pathways was investigated by using platelets from clopidogrel-treated subjects, 6 healthy volunteers (2 females and 4 males) who received 75 mg of clopidogrel daily for 7 days. Blood was taken and various platelet receptor signaling pathways were analyzed before treatment, after 7 days of medication, and 4 weeks after treatment had ceased. Platelet tests included the analysis of aggregation, rapid calcium influx, calcium mobilization from intracellular stores, adenylyl cyclase, and phosphorylation of vasodilator-stimulated phosphoprotein (VASP). The data indicate that clopidogrel does not affect those platelet ADP receptors coupled to cation influx (P2X₁ ADP receptors) or calcium mobilization (P2Y₁ ADP receptors). In contrast, clopidogrel treatment specifically impairs the ADP receptor coupled to G_i/adenylyl cyclase (P2Y_{AC} ADP receptors). Clopidogrel abolishes the inhibitory P2Y_{AC} receptor–mediated ADP effects on prostaglandin E₁–stimulated, cAMP-dependent phosphorylation of VASP without affecting epinephrine, thrombin, and thromboxane signaling. VASP phosphorylation is known to be closely correlated with the inhibition of platelet and fibrinogen receptor (glycoprotein IIb/IIIa) activation. Therefore, inhibition of the platelet P2Y_{AC} ADP receptor and its intracellular signaling, including decreased VASP phosphorylation, is suggested as a molecular mechanism of clopidogrel action. (*Arterioscler Thromb Vasc Biol.* 1999;19:2007–2011.)

Key Words: platelet inhibition ■ purinergic receptors ■ vasodilator-stimulated phosphoprotein

Increased platelet activation and aggregation are central to the pathophysiology of acute and chronic arterial vascular diseases. This concept has gained broad acceptance since platelet inhibitors have been proven as effective agents for the treatment of both chronic and acute diseases of the arterial vessel wall.^{1–6} Platelets are activated by numerous agents and conditions, but ADP is thought to play a key role in the development of arterial thrombosis.^{7,8} Recently, long-term administration of clopidogrel to patients with atherosclerotic vascular disease was shown to be more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction, and vascular death.⁹ Clopidogrel and the chemically related ticlopidine are thienopyridines that selectively and specifically interfere with ADP-mediated platelet activation.^{5,10} In contrast to ticlopidine, which may cause neutropenia, clopidogrel appears to be a safe and well-tolerated drug.⁹ Thienopyridines are inactive in-vitro, require in vivo metabolism, and cause an irreversible inhibition of platelet function. However, the mechanism of thienopyridine action is not well established owing to the limited understanding of platelet ADP receptors and their intracellular signaling.^{11,12}

Very recently, several laboratories, including those of our own groups, provided evidence for the existence of 3 distinct human platelet ADP receptors, which mediate ADP-caused cation influx (P2X₁ receptor), calcium mobilization (P2Y₁ receptor), and adenylyl cyclase inhibition (P2Y_{AC} receptor).^{13–20} Earlier studies with ticlopidine and clopidogrel had suggested that thienopyridines do not inhibit the ADP receptor pathways coupled to calcium influx and mobilization but instead, prevent the inhibitory ADP effects on adenylyl cyclase stimulation in platelets of different species.^{10,15,16,21–25} In the current study, we addressed the question as to which of the 3 now pharmacologically defined human platelet ADP receptors is affected by a clinically used clopidogrel dosage. Moreover, we analyzed the effect of clopidogrel on ADP-regulated intracellular signaling and protein phosphorylation. These studies were made possible because of the recent development of phosphorylation-specific monoclonal antibodies that allow quantitative analysis of vasodilator-stimulated phosphoprotein (VASP) phosphorylation in intact human platelets.²⁶ Previously, we showed that NO- and prostaglandin-stimulated phosphorylation of VASP, a

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Effect of Clopidogrel Treatment on Platelet Aggregation and cAMP

	Before Treatment	During Treatment	After Treatment	n
Relative aggregation, stimulated with				
ADP	100	10.92±5.25	90.19±35.28	5
U-46619	100	66.35±39.87	69.68±29.81	5
ADP/epinephrine	100	0.42±8.78	70.22±37.57	3
Relative cAMP content, stimulated with				
Unstimulated	0.87±0.16	1.06±0.40	0.97±0.28	5
PGE ₁	6.21±1.55	7.31±4.18	7.31±2.84	5
PGE ₁ /ADP	2.32±0.54	7.66±4.06	1.86±0.22	5
Unstimulated	0.89±0.15	0.99±0.51	0.74±0.26	5
PGE ₁	6.40±1.62	5.80±2.02	5.04±1.15	5
PGE ₁ /epinephrine	1.69±0.78	1.99±0.36	1.33±0.30	5

Values are mean±SEM. Platelet aggregation and cAMP levels were analyzed using platelets from volunteers before, during (after 7 days), and after (after 4 weeks) of clopidogrel treatment. Aggregation was stimulated with either 20 μ mol/L ADP, 5 μ mol/L U-46619, or a combination (2.5 μ mol/L ADP+3 μ mol/L epinephrine). Aggregation responses are relative to the aggregation before treatment. cAMP levels were stimulated with either 30 nmol/L PGE₁ or combinations (30 nmol/L PGE₁+5 μ mol/L ADP; 30 nmol/L PGE₁+55 μ mol/L epinephrine) as described in "Methods." cAMP levels are relative to those detected in platelets incubated with ADP alone. Data represent the means of experiments (number of volunteers) as indicated.

cytoskeleton/integrin-associated protein present at high concentrations in human platelets,^{27–30} is closely correlated with the inhibition of platelet and fibrinogen receptor (glycoprotein [GP] IIb/IIIa) activation.³⁰ This study now demonstrates that clopidogrel selectively impairs the human platelet P2Y_{AC} ADP receptor and its inhibitory effect on prostaglandin E₁-stimulated, cAMP-mediated VASP phosphorylation.

Methods

Volunteers

Six healthy volunteers (2 female and 4 male, with an age range of 23 to 48 years) who were not take any platelet-affecting drugs entered this study after their written, informed consent was obtained and after approval of this study by the ethics committee of our university. The volunteers took 75-mg clopidogrel tablets (provided by Sanofi and Bristol-Myers Squibb) daily for 7 days. Blood samples were taken before treatment, after 7 days of treatment, and 4 weeks after treatment had been discontinued. Blood counts of the volunteers were monitored throughout the study and were essentially unaltered.

Platelet Preparation, Platelet Aggregation, Platelet Calcium, and cAMP Regulation

Platelet-rich plasma and washed platelets were prepared from whole human blood, and platelet aggregation was determined with the aggregometer PAP-4 (Biodata) as previously described.¹⁵ Aggregation responses were determined in 0.3-mL samples of platelet-rich plasma. Aggregation was stimulated with either 20 μ mol/L ADP, 5 μ mol/L of the thromboxane analogue U-46619 (9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin F_{2 α}), or the combination of 2.5 μ mol/L ADP and 3 μ mol/L epinephrine. Platelet calcium and cAMP responses were determined as described in detail previously.¹⁵ For cAMP measurements, 0.3-mL aliquots of platelet suspension were incubated for 3 minutes in siliconized Eppendorf caps with either ethanol, 5 μ mol/L ADP, 30 nmol/L prostaglandin E₁ (PGE₁), or 30 nmol/L PGE₁ plus 5 μ mol/L ADP. The cAMP determination was performed using the Amersham Biotrak cAMP radioimmunoassay kit. Calcium responses were determined with aspirin-treated, washed, human platelets. The cells were loaded with 4 μ mol/L fura 2-AM and washed with a calcium-free buffer. Experiments

were performed using a Perkin-Elmer LS-50 fluorometer. Platelets were stimulated with 1 μ mol/L ADP or 1 μ mol/L U-46619 in the presence of 1 mmol/L Ca²⁺ or 4 mmol/L EGTA, respectively.

Western Blot Analysis of VASP Phosphorylation

The extent of VASP phosphorylation was determined in platelets obtained from platelet-rich plasma, which either was left untreated or incubated for 3 minutes with 5 μ mol/L ADP, 55 μ mol/L epinephrine, 30 nmol/L PGE₁, combinations thereof (30 nmol/L PGE₁+5 μ mol/L ADP; 30 nmol/L PGE₁+55 μ mol/L epinephrine), or vehicle alone. Platelets were then sedimented by centrifugation, the supernatant plasma was rapidly removed, and the platelet pellet was solubilized in a hot, SDS-containing stop solution. Platelet proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted on nitrocellulose, and analyzed for the extent of VASP serine 239 phosphorylation by the monoclonal antibody 16C2 as described.²⁶

Results

Platelet Aggregation

Clopidogrel treatment caused the nearly complete inhibition of ex vivo ADP-stimulated platelet aggregation (the Table). This treatment also abolished the ex vivo platelet aggregation in response to the combination of ADP and epinephrine at concentrations that, when used alone, did not activate platelets (the Table). The ADP-induced shape change, U-46619 (a stable thromboxane A₂ analogue)-stimulated aggregation, and thrombin-stimulated aggregation were not significantly inhibited (the Table; shape change and thrombin data not shown). A small inhibitory effect on thromboxane-induced aggregation may have been due to the secondary inhibition of ADP released by thromboxane-activated platelets. Platelet aggregation in response to ADP was essentially normal 4 weeks after clopidogrel treatment was discontinued (the Table). In 1 of our volunteers (a 23-year-old healthy male of normal weight), clopidogrel had little effect on ADP-stimulated platelet aggregation and on the other 5, platelet aggregation was analyzed in this study

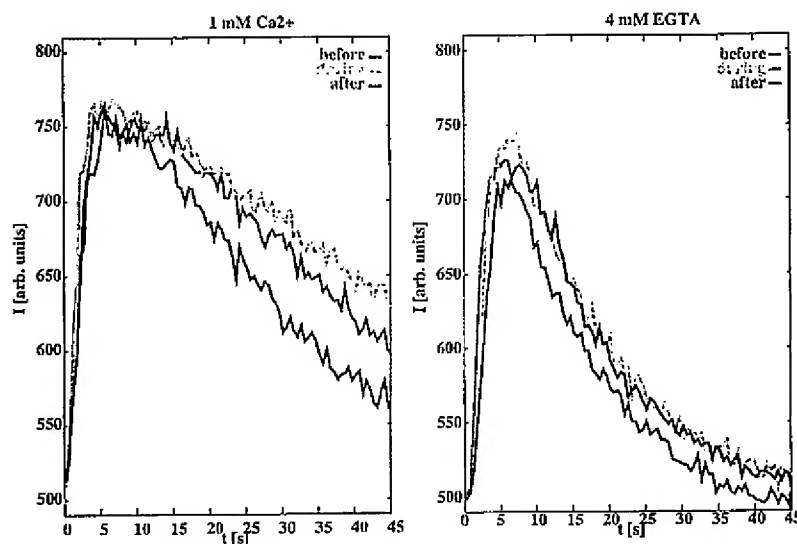


Figure 1. ADP-stimulated calcium responses of clopidogrel-treated platelets. Fura 2-loaded, washed, human platelets were stimulated with $1 \mu\text{mol/L}$ ADP in the presence of 1 mmol/L CaCl_2 or 4 mmol/L EGTA in the buffer medium as indicated. The traces shown are the responses obtained from the same individual before, during (7 days), and after (4 weeks) treatment with clopidogrel. Calcium experiments were performed with the platelets of 2 volunteers, the results being virtually identical for both. arb. indicates arbitrary.

(data not shown). Interestingly, a regular treatment with ticlopidine was also essentially ineffective in this individual (data not shown). This volunteer had no obvious blood and platelet abnormalities and was not included in our further data analyses. The frequency and mechanisms of impaired clopidogrel (and ticlopidine) responses in some individuals need further investigation.

Stimulated-Platelet Calcium Responses

In agreement with our previous study with ticlopidine,¹⁵ platelet calcium responses evoked by ADP or U-46619 were not affected by clopidogrel treatment. Neither calcium influx nor mobilization of intracellularly stored calcium ions was affected by thienopyridines¹⁵ (Figure 1).

Platelet cAMP Content

Clopidogrel treatment did not significantly alter the basal and PGE_1 -stimulated cAMP content in platelets. In contrast, the inhibitory effects of ADP (but not those of epinephrine) on PGE_1 -stimulated cAMP levels were abolished by clopidogrel treatment (the Table).

VASP Phosphorylation

Both ADP and epinephrine inhibited PGE_1 -stimulated VASP phosphorylation at serine 157 (data not shown; detected by the phosphorylation-induced shift of VASP from the 46- to the 50-kDa form³⁰) and at serine 239 (detected by the phosphorylation-specific monoclonal antibody 16C2²⁶) as demonstrated in Figures 2 and 3. Clopidogrel treatment did not alter basal and PGE_1 -stimulated VASP phosphorylation but strongly attenuated the inhibitory effect of ADP on PGE_1 -stimulated VASP phosphorylation (Figures 2 and 3). All ADP responses were essentially restored 4 weeks after treatment was discontinued. The inhibitory effects of epinephrine on PGE_1 -induced VASP phosphorylation were not affected by clopidogrel treatment (Figure 3).

Discussion

The data presented show that a clinically effective dose of clopidogrel⁹ selectively inhibits ADP-stimulated platelet aggregation and impairs the inhibitory ADP effects on platelet cAMP levels and cAMP-induced VASP phosphorylation. In

agreement with earlier studies,^{21–25} clopidogrel inhibits the effects of ADP on adenylyl cyclase stimulation without affecting ADP-induced calcium influx or calcium mobilization in platelets tested *ex vivo*. As summarized in Figure 4, clopidogrel thus inhibits the G_i protein-coupled P2Y_{AC} ADP receptor but neither the cation channel-coupled P2X_1 ADP receptor nor the G_q protein-coupled P2Y_1 ADP receptor, which were only very recently identified and characterized.^{12–20} Interestingly, clopidogrel did not affect the inhibitory, also G_i protein-mediated, effect of epinephrine on platelet cAMP levels and VASP phosphorylation. These findings strongly suggest that clopidogrel impairs the platelet P2Y_{AC} ADP receptor at the receptor level directly or at a level preceding G_i protein(s). This conclusion is also supported by earlier observations that clopidogrel reduces the number of binding sites for 2-methylthio-ADP in human and rat platelets.^{23,31} The time course of clopidogrel effects (although not extensively studied here) appears to be similar to that of ticlopidine (the Table and other data not shown). The practically irreversible anti-ADP effects of ticlopidine and clopidogrel (which are inactive *in vitro*) reach their maximum within 3 to 4 days of treatment and are completely gone

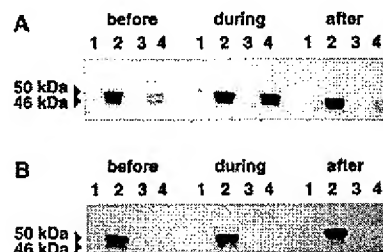


Figure 2. Western blots showing the effect of clopidogrel on PGE_1 -, ADP-, and epinephrine-regulated VASP serine 239 phosphorylation in platelets. PGE_1 -, ADP-, and epinephrine-regulated VASP serine 239 phosphorylation was analyzed as described in Methods by using platelets obtained from volunteers before, during (7 days), and after (4 weeks) clopidogrel treatment. A, Lane 1, control; lane 2, PGE_1 treated; lane 3, ADP treated; and lane 4, ADP- and PGE_1 -treated platelets. B, Lane 1, control; lane 2, PGE_1 treated; lane 3, epinephrine treated; and lane 4, epinephrine- and PGE_1 -treated platelets. Western blot data of 1 experiment representative of the results obtained with platelets from 2 volunteers are shown.

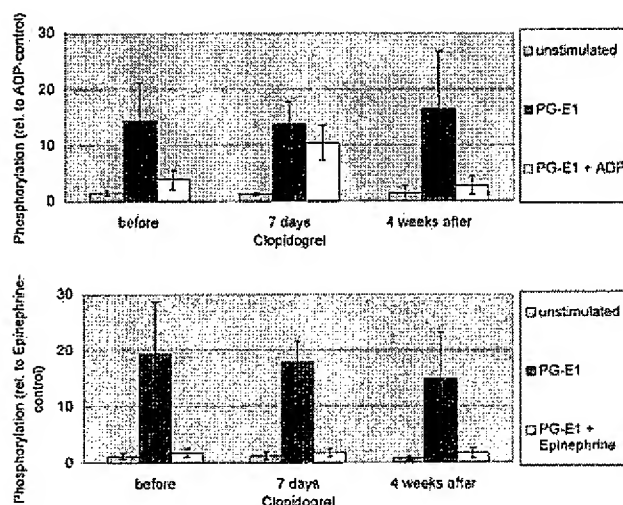


Figure 3. Clopidogrel effects on PGE₁-, ADP-, and epinephrine-regulated VASP serine 239 phosphorylation in platelets. PGE₁-, ADP-, and epinephrine-regulated VASP serine 239 phosphorylation was analyzed as described (see Methods and Figure 2) by using platelets obtained from volunteers before, during (7 days), and after (4 weeks) clopidogrel treatment. VASP serine 239 phosphorylation data (reported relative to the extent seen in platelets treated with ADP or epinephrine alone) are the mean \pm SEM of the results obtained with platelets from 5 volunteers.

within 3 to 4 weeks after discontinuation of treatment.^{5,10,15} The specificity of clopidogrel, which (under the conditions used) impairs the P2Y_{AC} ADP receptor only without detectable effect on many other platelet receptors involved in the activation or aggregation of platelets, is remarkable.

The mechanisms of G protein-coupled receptor regulation of platelet GP IIb/IIIa activation and ultimately aggregation have not been fully elucidated²⁹ but appear to involve more than 1 intracellular pathway and distinct regulatory molecules, as summarized in Figure 4. Activation of the platelet P2Y_{AC} ADP receptor by ADP liberates the G_i protein subunits α_{Gi} and $\beta\gamma$, which couple to independent signaling events (Figure 4). Subunit α_{Gi} decreases platelet cAMP levels and (among various cAMP-regulated events, including inhibition of ADP receptor activation of phospholipase C³²) reduces the level of phospho-

VASP. Phosphorylation of VASP in response to cAMP-elevating agents is closely correlated with the inhibition of GP IIb/IIIa.^{29,30} In vivo intact endothelium serves as a source of cAMP-elevating factors such as prostacyclin, which stimulate VASP phosphorylation and inhibit platelet aggregation. Indeed, endothelium-dependent platelet VASP phosphorylation has been demonstrated in platelet-endothelial cell cocultures and in the intact coronary system.^{33,34} The important regulatory role of VASP in platelet activation/aggregation is supported by very recent data obtained with platelets from VASP-deficient mice. VASP-deficient murine platelets, when compared with wild-type murine platelets, displayed an impaired cyclic nucleotide-mediated inhibition of aggregation and an enhanced thrombin- and collagen-induced integrin $\alpha_{IIb}\beta_3$ activation.^{35,36} Other recent in vitro data suggest that the platelet P2Y_{AC} and P2Y₁ ADP receptors and their G_i- or G_q-coupled signaling are both required but alone are insufficient to mediate ADP-evoked platelet aggregation.^{16,17,20} For example, platelet aggregation was not induced by 10 μ M ADP in the presence of the P2Y_{AC} blocker ARL 66096 but occurred when an additional 1 μ M epinephrine was used.¹⁷ Similarly, 2-methylthio-ADP-induced platelet aggregation was inhibited by the P2Y₁ blocker A3P5PS but was restored by the addition of 2.5 μ M serotonin.¹⁶ In our present experiments (the Table), the P2Y_{AC} blocker clopidogrel not only inhibited ADP (20 μ M/L)-evoked aggregation but also the aggregation response by the combination of low-dose (2.5 μ M/L) ADP and 3 μ M/L epinephrine. Whereas 10 to 20 μ M/L ADP alone is sufficient to induce aggregation¹⁷ (see also the Table), low-dose (2.5 μ M/L) ADP concentrations (which alone did not cause aggregation) were chosen to demonstrate the synergism between ADP and epinephrine and its possible sensitivity to clopidogrel treatment. Our data (the Table) demonstrate that the P2Y_{AC} receptor is required for the synergism between ADP and epinephrine with respect to aggregation when low concentrations of ADP are used. Our present data with clopidogrel are in full agreement with the suggestion^{16,17,20} that both the P2Y_{AC} ADP receptor and the P2Y₁ ADP receptor are required and essential for ADP-induced platelet aggregation. The relative contribution of each of these 2 ADP receptors in mediating the in vivo effects of ADP may depend on

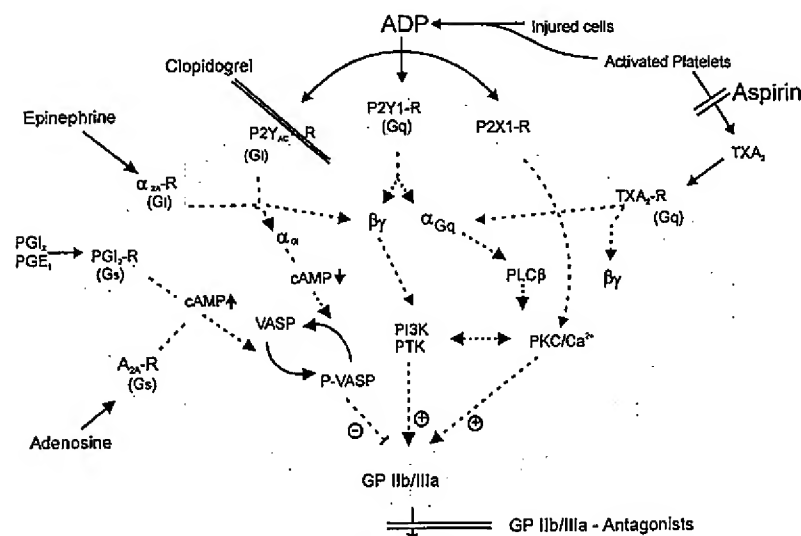


Figure 4. Sites of platelet inhibition by clopidogrel, aspirin, and GP IIb/IIIa antagonists. Clopidogrel selectively impairs the molecularly not yet cloned P2Y_{AC} ADP receptor without affecting ADP-gated cation channels (P2Y₁ ADP receptor), the phospholipase C (PLC)/G_q protein-coupled P2Y₁ ADP receptor, or the G_i protein-coupled epinephrine (α_{2A} -R) adrenergic receptor (α_{2A} -R). G_i protein-coupled receptors inhibit and G_q protein-coupled receptors promote cAMP-mediated phosphorylation of VASP (P-VASP), which is known to be closely correlated with the inhibition of aggregation and fibrinogen receptor (GP IIb/IIIa) activation.²³ The detailed mechanisms of GP IIb/IIIa activation and inhibition have not been fully elucidated at the molecular level but involve multiple receptors and signaling events as discussed in the text. Aspirin inhibits the generation of platelet thromboxane A₂ (TXA₂) and thereby its receptor (TXA₂-R)-mediated events. PI3K indicates phosphoinositide 3-kinase; PTK, phosphorylating kinase; and PKC, protein kinase C.

the local concentrations of ADP and the regulation of these ADP receptors by other signaling pathways. In this respect, it is of interest to note that the P2Y₁ ADP receptor linked to calcium mobilization is strongly inhibited by both cAMP- and cGMP-regulated pathways.³² P2Y_{AC} receptor-mediated inhibition of VASP phosphorylation may be an important component of ADP-stimulated platelet aggregation in vivo. However, activation of G_i proteins by ADP may also liberate the $\beta\gamma$ subunit complex, which is known to activate the C protein kinases, phosphoinositol 3-kinase, and the phosphotyrosine kinases,³⁷ all of which are thought to be linked to GP IIb/IIIa activation.²⁹ Clearly, elucidation of the molecular mechanisms of platelet activation and aggregation mediated by G protein-coupled receptors needs further investigation. In conclusion, the clinical efficacy of clopidogrel⁹ and our present data indicate an important role of the platelet P2Y_{AC} ADP receptor in mediating the in vivo effects of ADP that are associated with arterial thrombosis in patients at high risk for arterial cardiovascular complications.

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Exhibit 35

Clopidogrel for Coronary Stenting

Response Variability, Drug Resistance, and the Effect of Pretreatment Platelet Reactivity

Paul A. Gurbel, MD; Kevin P. Bliden, BS; Bonnie L. Hiatt, MD; Christopher M. O'Connor, MD

Background—Clopidogrel is administered to prevent stent thrombosis; however, the uniformity of platelet inhibition after treatment and the influence of pretreatment reactivity on drug response have not been described.

Methods and Results—Platelet aggregation (5 and 20 $\mu\text{mol/L}$ ADP), the activation of glycoprotein IIb/IIIa (PAC-1 antibody), and the expression of P-selectin were measured in patients undergoing elective coronary stenting ($n=96$) at baseline and at 2 hours, 24 hours, 5 days, and 30 days after stenting. All patients received aspirin (325 mg). Clopidogrel (300 mg) was administered in the catheterization laboratory and followed by 75 mg daily. There was marked interindividual variability in drug response as measured by all markers that showed a normal distribution. Resistance, defined as baseline aggregation (%) minus posttreatment aggregation (%) $\leq 10\%$ by 5 $\mu\text{mol/L}$ ADP, was present in 31% and 15% of patients at 5 and 30 days, respectively. Patients with the highest pretreatment platelet reactivity remained the most reactive at 24 hours after treatment ($P<0.0001$).

Conclusions—Interindividual variability in the platelet inhibitory response from clopidogrel occurs in patients undergoing elective coronary stenting. Patients with high pretreatment reactivity are least protected. Alternative pharmacological strategies and the association of adverse ischemic events should be investigated in these patients. (*Circulation*. 2003; 107:2908-2913.)

Key Words: drugs ■ platelets ■ stents

Clopidogrel with aspirin is the regimen of choice to prevent stent thrombosis.¹ The CURE study (Clopidogrel in Unstable angina to prevent Recurrent Events) showed that combination clopidogrel and aspirin antiplatelet therapy reduces ischemic events compared with aspirin therapy alone.² These findings are consistent with those of the CAPRIE study (Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events), which showed superior reduction in ischemic events with clopidogrel therapy compared with aspirin, and which may be explained in part by aspirin resistance.³ However, the uniformity of inhibition after clopidogrel therapy and the incidence of drug resistance has not been investigated extensively. Interindividual variability in response to clopidogrel may affect clinical outcomes.⁴

We studied the individual responses to clopidogrel therapy in patients undergoing elective coronary artery stenting by measuring platelet aggregation and other markers of platelet activation by flow cytometry for 30 days after the procedure.⁵ The frequency of drug resistance is reported. We also studied the influence of pretreatment platelet reactivity on drug response.

Methods

This study was approved by the Investigational Review Board. Consecutive patients undergoing elective coronary stenting were

enrolled after giving informed consent. All ages were included. The exclusion criteria were a history of bleeding diathesis, acute myocardial infarction within 48 hours, cerebrovascular event within 3 months, illicit drug or alcohol abuse, prothrombin time >1.5 times control, platelet count $<100\,000/\text{mm}^3$, hematocrit $<25\%$, creatinine >4.0 mg/dL, and thienopyridine or glycoprotein (GP) IIb/IIIa use before the procedure.

Per protocol, GP IIb/IIIa inhibitors were not given. Clopidogrel (300 mg) was given to all patients in the catheterization laboratory after successful coronary artery stent implantation followed by 75 mg daily for 30 days. In addition, all patients had received at least 81 mg of aspirin for 7 days before the procedure ($>90\%$ received 325 mg) and were administered 325 mg on the day of the procedure and daily thereafter. Heparin to achieve an activated clotting time >300 seconds was administered as a bolus to all patients in the catheterization laboratory immediately before stenting.

Blood Sampling

Blood was collected in evacuated container tubes containing 3.8% trisodium citrate that were filled to capacity and then inverted 3 to 5 times for gentle mixing. Samples were obtained before clopidogrel administration (baseline) and at 2 hours, 24 hours, 5 days, and 30 days after stenting.

Platelet Aggregation

The blood-citrate mixture was centrifuged at 1200g for 2.5 minutes. The resulting platelet-rich plasma was kept at room temperature for use within 1 hour. The platelet count was determined in the

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platelet-rich plasma sample and adjusted to $3.5 \times 10^8/\text{mL}$ with homologous platelet-poor plasma. Platelets were stimulated with ADP (5 and 20 $\mu\text{mol/L}$), and aggregation was assessed as described previously with a Chronolog Lumi-Aggregometer (model 560-Ca) with the AggroLink software package.⁶ Platelet aggregation was expressed as the maximal percent change in light transmittance from baseline, with platelet-poor plasma used as a reference. Curves were analyzed according to accepted standards.⁷

Flow Cytometry

The surface expression of platelet receptors was determined by flow cytometry with monoclonal antibodies. Briefly, the blood-citrate mixture (50 μL) was diluted with 450 μL of Tris buffered saline (10 mmol/L Tris, 0.15 mol/L sodium chloride) and mixed by inverting an Eppendorf tube gently 2 times. The corresponding antibody was then added (5 μL) and incubated at room temperature for 30 minutes. After incubation, 400 μL of 2% buffered paraformaldehyde was added for fixation. The samples were analyzed on a Becton Dickinson FACScan flow cytometer set up to measure fluorescent light scatter as described previously.⁸ All parameters were collected with four-decade logarithmic amplification. The data were collected in list-mode files and then analyzed. The PAC-1 antibody (Becton Dickinson) binds only to the active $\alpha_{\text{IIb}}\beta_3$ receptor, and therefore the total amount of $\alpha_{\text{IIb}}\beta_3$ is not determined.⁹ PAC-1 was expressed as log mean fluorescence intensity. P-selectin (Pharmingen) was measured after stimulation with 200 $\mu\text{mol/L}$ ADP and is expressed as percent positivity (ie, the percentage of platelets positive for the antibody) as described previously.¹⁰ The dose of agonist was chosen on the basis of data reporting maximum expression of P-selectin induced by 100 $\mu\text{mol/L}$ ADP in the absence of an ADP blocker.⁵

Drug Resistance Definition

Drug resistance was defined as an absolute difference between baseline aggregation and posttreatment aggregation (Δ aggregation [%]) of 10% or less with 5 $\mu\text{mol/L}$ ADP used as the agonist. Because Δ aggregation (%) = baseline aggregation (%) - posttreatment aggregation (%), a negative Δ aggregation would indicate poststent platelet reactivity greater than baseline, and a positive Δ aggregation would indicate platelet inhibition.

Statistical Analysis

The responders and nonresponders were compared with *t* tests. Standard regression analysis was used to correlate 5 and 20 $\mu\text{mol/L}$ ADP-induced aggregation and 5- and 30-day aggregation and P-selectin expression (Statistica software).

To assess the effect of pretreatment reactivity on drug response, patients were divided into high, moderate, and low baseline reactivity.¹¹ Two separate analyses were performed on the basis of aggregation and P-selectin expression. For 5 $\mu\text{mol/L}$ ADP-induced aggregation, high reactivity was defined as percent aggregation >70%; moderate, 60% to 70%; and low, <60%. For P-selectin, high reactivity was defined as percent positivity >50%; moderate, 40% to 50%; and low, <40%. Comparisons were made between groups by 1-way ANOVA (Statistica software). The Wilks-Shapiro test was used to assess conformity with a normal distribution. Curves were plotted of the best fit to a normal distribution by Statistica software. Given the normal distribution of data, the mean \pm SD and mean \pm SE were used. $P < 0.05$ was considered significant.

Results

Patient Data

Ninety-six patients had complete platelet studies performed at baseline, and of these patients, 92 had adequate poststent samples. The patient demographics on these 92 patients are shown with respect to the response to 5 $\mu\text{mol/L}$ ADP-induced aggregation at day 5 in the Table. The patients were elderly, and most were males. Multiple cardiovascular risk factors

Demographics Based on Response to 5 $\mu\text{mol/L}$ ADP at Day 5

Parameter	Responders	Nonresponders	P
Sex, % male	63	50	NS
Age, y	66 \pm 12	69 \pm 10	NS
Weight, lb	190 \pm 40	197 \pm 36	NS
Smoking, %			
<6 Months ago	28	13	NS for all
>6 Months ago	38	38	
Never	44	50	
Previous infarction, %	26	44	NS
Previous PTCA, %	23	19	NS
Previous CABG, %	23	6	NS
Hypercholesterolemia, %	66	50	NS
Diabetes, %	42	38	NS
Family history, %	59	56	NS
Concomitant medications, %			
β -Blocker	68	50	NS
ACE inhibitor	57	81	0.08
Calcium channel antagonist	13	31	0.09
Statin	64	56	NS
Aspirin	100	100	NS
Procedural variables			
Stent length, mm	19.6 \pm 11.7	13.8 \pm 7.5	0.007
Minimal stent diameter, mm	3.0 \pm 0.4	3.1 \pm 0.5	NS
No. stents/patient	1.5 \pm 0.4	1.4 \pm 0.3	NS

CABG indicates coronary artery bypass graft; ACE, angiotensin-converting enzyme; and PTCA, percutaneous transluminal coronary angioplasty.

NS = $P > 0.1$.

were frequent. Concomitant drug use did not differ significantly between groups. A trend of higher calcium antagonist and ACE inhibitor use was observed in the nonresponders. Most patients were treated with 1 stent. There were no significant procedural differences between responders and nonresponders except for total stent length. Follow-up at 30 days revealed no cases of Q-wave myocardial infarction, stent thrombosis, target-vessel revascularization, cerebrovascular ischemic events, or death.

Platelet Aggregation

Histograms of the response to clopidogrel are shown in Figures 1 and 2. Baseline aggregation to 5 and 20 $\mu\text{mol/L}$ ADP was $62 \pm 18\%$ and $83 \pm 21\%$, respectively. The response to 5 and 20 $\mu\text{mol/L}$ ADP showed a shift to the right between 2 and 24 hours after treatment, which indicates increased platelet inhibition. Aggregation by 5 $\mu\text{mol/L}$ ADP was maximally inhibited by 24 hours ($P < 0.05$ compared with baseline). Platelet aggregation was $58 \pm 22\%$ at 2 hours, $37 \pm 22\%$ at 24 hours, $32 \pm 18\%$ at 5 days, and $31 \pm 15\%$ at 30 days. At 2 hours after stenting, 63% of patients met the definition of resistance and platelet reactivity was greatest, with 42% of patients having greater aggregation than at baseline. At 24 hours, resistance fell to 31%, and 24% of patients still had greater aggregation than at baseline. No

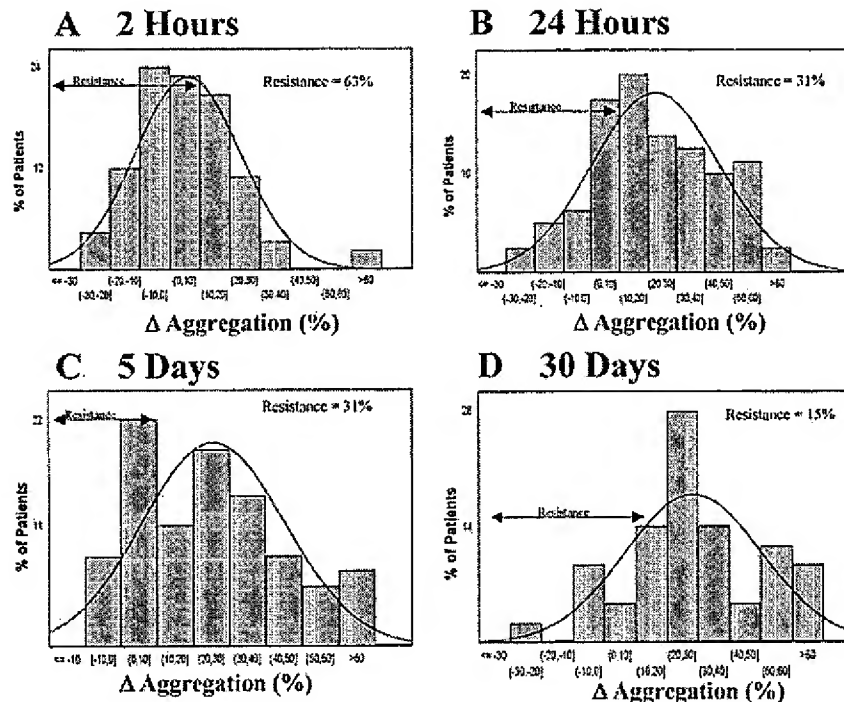


Figure 1. Relationship between frequency of patients and absolute change in aggregation (Δ Aggregation [%]) in response to 5 μ mol/L ADP at 2 hours (A), 24 hours (B), 5 days (C), and 30 days (D) after stenting. Δ Aggregation (%) is defined as baseline aggregation (%) minus posttreatment aggregation (%). Resistance, as defined herein, is Δ Aggregation (%) $\leq 10\%$. Resistance is present in those patients subtended by double-headed arrow. Curves represent normal distribution of data and were created by Statistica software.

further changes were seen at 5 days, when resistance was observed in 31%. However, at 30 days after stenting, the incidence of resistance fell to 15%, but 11% still had aggregation greater than baseline.

The response to 20 μ mol/L ADP showed a similar pattern. Aggregation was $80 \pm 24\%$ at 2 hours and fell to $60 \pm 25\%$ at 24 hours ($P < 0.05$ compared with baseline). At 5 days, aggregation remained stable ($57 \pm 23\%$), with a nonsignificant decrease at 30 days ($52 \pm 14\%$). A Δ aggregation of 10% or less was present in 53% of patients at 2 hours, 35% at 24

hours, 32% at 5 days, and 21% at 30 days. The correlation between the 5- and 20- μ mol/L ADP aggregation response was strong ($r = 0.6$).

Correlation of Responses at 5 and 30 Days

A strong correlation was observed between the 5- and 30-day responses to 5 μ mol/L ADP ($r = 0.8$). Moreover, strong correlations were also observed between 5- and 30-day responses for 20 μ mol/L ADP ($r = 0.8$) and P-selectin ($r = 0.7$).

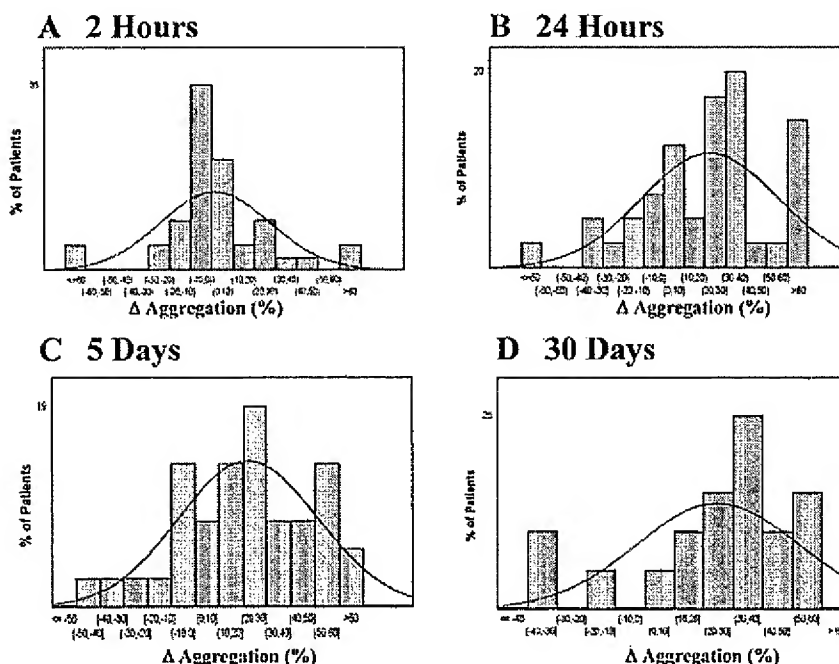


Figure 2. Relationship between frequency of patients and absolute change in aggregation (Δ Aggregation [%]) in response to 20 μ mol/L ADP at 2 hours (A), 24 hours (B), 5 days (C), and 30 days (D) after stenting. Δ Aggregation (%) is defined as baseline aggregation (%) minus posttreatment aggregation (%).

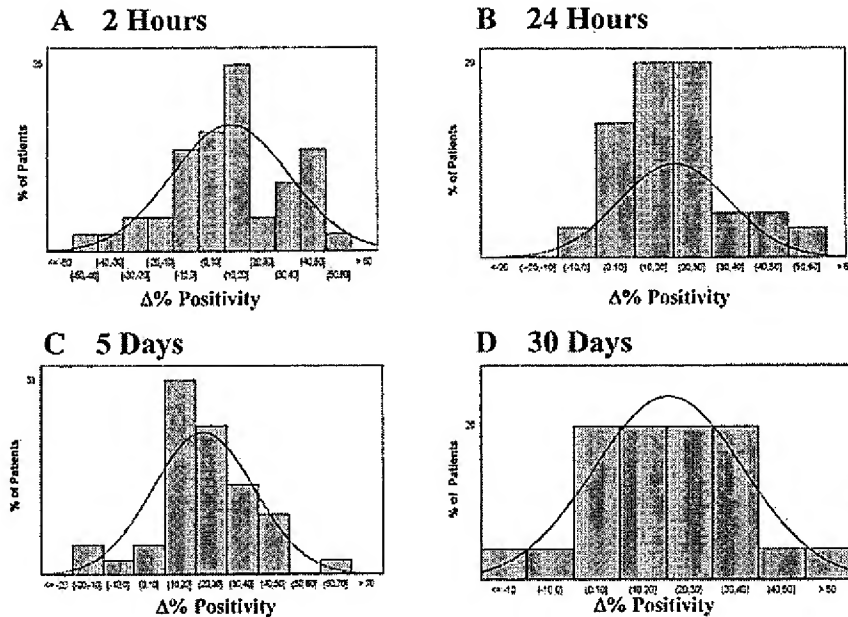


Figure 3. Relationship between frequency of patients and absolute change in % positivity of stimulated P-selectin expression ($\Delta\%$ Positivity) at 2 hours (A), 24 hours (B), 5 days (C), and 30 days (D) after stenting. $\Delta\%$ Positivity is defined as baseline positivity (%) minus posttreatment positivity (%).

Platelet Receptor Expression

P-Selectin

Baseline stimulated P-selectin expression was $45 \pm 16\%$ and fell over 24 hours, as indicated by a shift in the curve to the right (Figure 3). Maximum inhibition of P-selectin expression occurred within 24 hours ($24 \pm 13\%$; $P < 0.05$ compared with baseline) and was unchanged at 5 days ($22 \pm 13\%$) and 30 days ($23 \pm 10\%$), as observed in the aggregation studies. An absolute change in percent positivity of 10% or less was observed in 44% of patients at 2 hours, 25% at 24 hours, 12% at 5 days, and 29% at 30 days, which again suggests resistance to the standard clopidogrel regimen.

PAC-1

PAC-1 binding showed similar response variability (Figure 4). Baseline expression was 14.9 ± 13.1 , and inhibition of the

expression of active GP IIb/IIIa was maximal within 24 hours (8.5 ± 5.1 ; $P < 0.05$ compared with baseline). No significant changes as compared with 24 hours were observed at 5 days (8.1 ± 3.7) or 30 days (8.6 ± 5.3).

Effect of Pretreatment Platelet Reactivity on Drug Response

High pretreatment reactivity, defined by the response to $5 \mu\text{mol/L}$ ADP, was present in 31 patients, moderate reactivity in 25 patients, and low reactivity in 40 patients. High-reactivity patients had a greater incidence of diabetes (71%; $P < 0.05$) than those with moderate (24%) and low (40%) reactivity. Patient weight, gender, age, statin use, smoking history, incidence of hyperlipidemia, history of prior infarction,

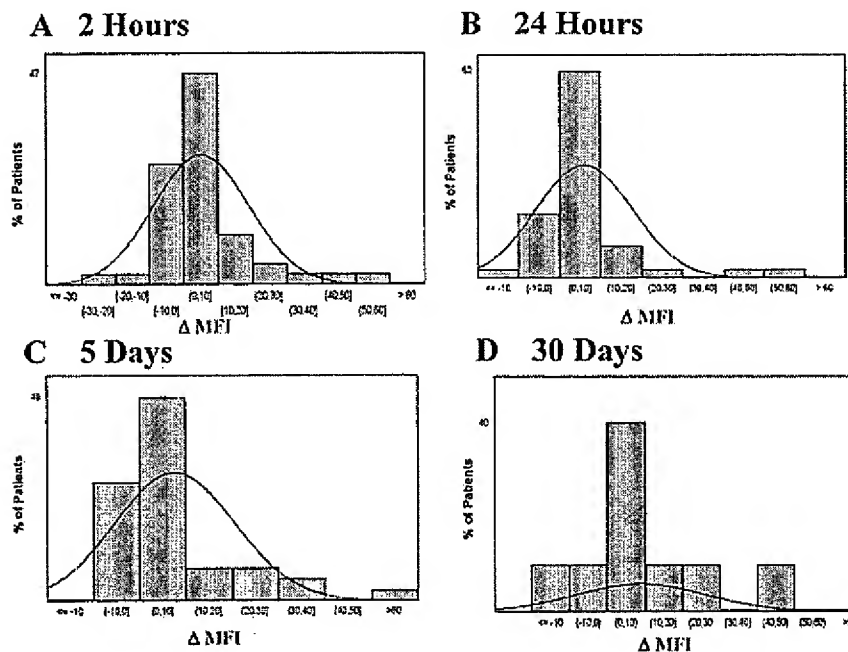


Figure 4. Relationship between frequency of patients and absolute change in mean fluorescence intensity (MFI) of PAC-1 binding (ΔMFI) at 2 hours (A), 24 hours (B), 5 days (C), and 30 days (D) after stenting. ΔMFI is defined as baseline MFI minus posttreatment MFI.

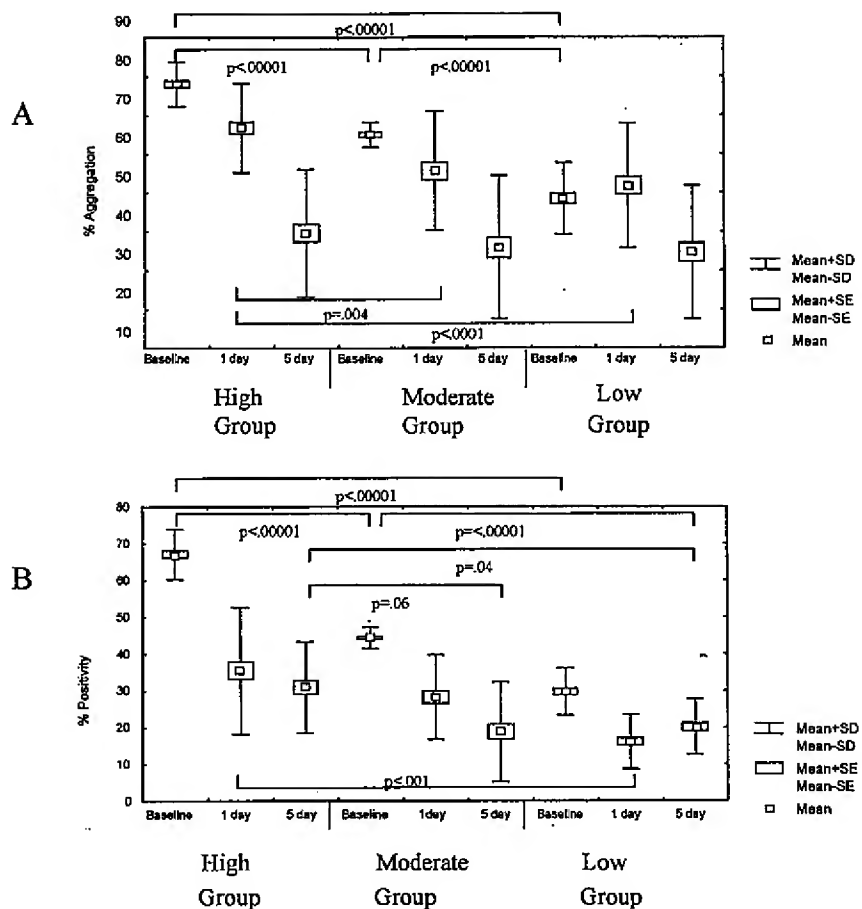


Figure 5. A, ADP-induced platelet aggregation ($5 \mu\text{mol/L}$ ADP) in high-, moderate-, and low-reactivity groups at baseline and at 1 and 5 days after clopidogrel therapy. High-reactivity patients were defined as pretreatment percent aggregation $>70\%$; moderate, 60% to 70% ; and low, $<60\%$. B, Stimulated P-selectin expression in high-, moderate-, and low-reactivity groups at baseline and at 1 and 5 days after clopidogrel therapy. High-reactivity patients were defined as pretreatment percent positivity $>50\%$; moderate, 40% to 50% ; and low, $<40\%$.

tion, total contrast load, procedure duration, and number of vessels treated were not significantly different between groups.

Platelet Aggregation

At baseline, by definition, the high-reactivity group had markedly greater reactivity ($78 \pm 6\%$) than the moderate ($65 \pm 3\%$; $P < 0.00001$) and low ($48 \pm 9\%$; $P < 0.00001$) groups (Figure 5A). At day 1 after stenting, the high-reactivity group continued to have the most reactive platelets ($67 \pm 11\%$; $P = 0.004$ versus moderate [$56 \pm 15\%$] and $P < 0.0001$ versus low [$52 \pm 15\%$]). By day 5, platelet reactivity by this marker was similar among groups.

P-Selectin Expression

At baseline, the high-reactivity group had greater expression ($67 \pm 7\%$) than the moderate ($44 \pm 3\%$; $P < 0.00001$) and low ($29 \pm 6\%$; $P < 0.00001$) groups (Figure 5B). The effect that pretreatment reactivity had on the inhibitory response to clopidogrel at day 1 was remarkably similar to findings with ADP-induced light-transmittance aggregometry. At day 1, patients with high pretreatment P-selectin expression remained the most reactive ($P < 0.001$ versus low reactivity). At day 5 of therapy, patients in the high-reactivity group had a trend to greater P-selectin expression than the moderate ($31 \pm 12\%$ versus $19 \pm 13\%$; $P = 0.06$) and low ($20 \pm 7\%$; $P = 0.04$) groups.

Discussion

The present study illustrates the variable platelet inhibitory response to the standard administered dose of clopidogrel. The platelet aggregation studies used 2 agonist concentrations that showed a strong correlation. In addition, platelet receptor expression showed similar findings. These uniform observations, irrespective of the methodology chosen to detect inhibition, strengthen our conclusions that the response to clopidogrel therapy is indeed heterogeneous and that drug resistance occurs. Our observations are in agreement with one other report of 18 patients with stable angina treated with the same clopidogrel regimen after coronary intervention.¹² Those investigators demonstrated variable inhibition of ADP-induced fibrinogen binding on day 2 after stenting.

Our definition of drug resistance was empirical because there have been no extensive reports on this subject among patients treated with clopidogrel. Clopidogrel inhibits aggregation in response to ADP, and therefore, we studied the response to 2 different concentrations of this agonist with light-transmittance aggregometry. Moreover, we assessed the expression of an established marker of platelet activation (P-selectin) in response to a maximal agonist concentration and studied the response of a sensitive platelet activation-dependent marker (PAC-1 binding) in nonstimulated blood.^{5,9}

The present study suggests that the maximum inhibitory response to a 300-mg loading dose followed by 75 mg/d occurs within 24 hours. These findings are consistent with

reports in healthy volunteers and in patients undergoing coronary stenting.^{13,14}

The response to clopidogrel appears to be patient specific. The robust correlations demonstrated that in most patients, the 30-day inhibitory response from clopidogrel was predicted by the 5-day response. Of equal importance, the present investigation also suggests that resistance to clopidogrel does not accrue over time.

The present study is the first to demonstrate that the level of platelet reactivity after the standard clopidogrel regimen for coronary stenting is critically dependent on the pretreatment reactivity. The present in vitro tests suggest that patients with the greatest pretreatment platelet activity have the least antithrombotic protection, particularly within the first 24 hours of therapy. The level of platelet reactivity has been correlated with adverse events by others.⁴ Moreover, an examination of P-selectin expression suggests that this relationship is true even after 5 days of therapy, when those patients with the greatest baseline expression of this activation-dependent receptor tended to be more reactive than those with baseline low or moderate expression. The present findings may help to explain why ticlopidine without a loading dose did not prevent stent thrombosis in the first 3 days after the procedure.¹⁵ The similar findings at 24 hours using 2 different established markers of platelet activity strengthen our conclusion that the response to clopidogrel therapy is indeed dependent on pretreatment reactivity. Previous investigations using aggregometry and P-selectin expression as markers of reactivity have shown that loading doses higher than 300 mg may enhance and accelerate platelet inhibition in patients undergoing coronary interventions.^{16–19} Similar strategies may particularly benefit patients with high pretreatment reactivity.

Limitations

The present study included patients undergoing elective coronary stenting, which is known to increase platelet reactivity.⁸ Because pretreatment reactivity affected the reactivity measured after antiplatelet therapy, postdrug platelet reactivity may be less in studies of healthy volunteers and in patients with stable coronary artery disease. Our definition of resistance involves the amplitude of maximal platelet aggregation and can be influenced by various factors, including inpatient variability. The current rates of stent thrombosis observed in elective stenting are much lower than the incidence of clopidogrel resistance in the present study, which suggests that our definition may be an overestimate or that resistance to clopidogrel is not a primary factor influencing stent thrombosis in these patients. However, the present data imply that nonresponders with high pretreatment reactivity may be at greatest risk.

In conclusion, the platelet inhibitory response to the standard dosing regimen of clopidogrel for coronary stenting is variable, follows a normal distribution, and appears stable over 30 days. Patients with high pretreatment reactivity are the least protected within the first 5 days of treatment. Further study is necessary to investigate the mechanisms of these findings and how they correlate with the occurrence of

ischemic events. The present work would also support further investigations to determine whether higher clopidogrel doses may overcome interindividual differences in drug response.

Acknowledgments

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Exhibit 36



European Medicines Agency
Evaluation of Medicines for Human Use

Doc.Ref.: EMEA/117561/2009

ASSESSMENT REPORT

FOR



International Nonproprietary Name: prasugrel

Procedure No. EMEA/H/C/000984

Assessment Report as adopted by the CHMP with
all information of a commercially confidential nature deleted.

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1. BACKGROUND INFORMATION ON THE PROCEDURE

1.1 Submission of the dossier

The applicant Eli Lilly Nederland B.V. submitted on 06 February 2008 an application for Marketing Authorisation to the European Medicines Agency (EMA) for Efient, through the centralised procedure under Article 3 (2) (a) of Regulation (EC) No 726/2004. The eligibility to the centralised procedure was agreed upon by the EMA/CHMP on 25 January 2007.

The legal basis for this application refers to:

A - Centralised / Article 8(3) / New active substance.

Article 8.3 of Directive 2001/83/EC, as amended - complete and independent application

The application submitted is a complete dossier composed of administrative information, complete quality data, non-clinical and clinical data based on applicants' own tests and studies and/or bibliographic literature substituting/supporting certain test(s) or study(ies).

Licensing status:

The product was not licensed in any country at the time of submission of the application.

The Rapporteur and Co-Rapporteur appointed by the CHMP were:

Rapporteur: Steffen Thirstrup Co-Rapporteur: Gonzalo Calvo Rojas

1.2 Steps taken for the assessment of the product

- The application was received by the EMA on 6 February 2008.
- The procedure started on 27 February 2008.
- The Rapporteur's first Assessment Report was circulated to all CHMP members on 19 May 2008. The Co-Rapporteur's first Assessment Report was circulated to all CHMP members on 20 May 2008.
- During the meeting 23-26 June 2008, the CHMP agreed on the consolidated List of Questions to be sent to the applicant. The final consolidated List of Questions was sent to the applicant on 26 June 2008.
- The applicant submitted the responses to the CHMP consolidated List of Questions on 21 July 2008.
- The Rapporteurs circulated the Joint Assessment Report on the applicant's responses to the List of Questions to all CHMP members on 9 September 2008.
- During the CHMP meeting on 22-25 September 2008, the CHMP agreed on a List of Outstanding Issues to be addressed in writing and in an oral explanation by the applicant and in addition CHMP agreed on questions to be addressed to a SAG-CVS.
- The applicant submitted the written responses to the CHMP List of Outstanding Issues on 16 October 2008.
- During a meeting of a SAG group on 30 October 2008, experts were convened to address questions raised by the CHMP.
- During the CHMP meeting on 17-20 November 2008, outstanding issues were addressed by the applicant during an oral explanation before the CHMP.
- During the meeting on 15-18 December 2008, the CHMP, in the light of the overall data submitted and the scientific discussion within the Committee, issued a positive opinion for granting a Marketing Authorisation to Efient on 18 December 2008. The applicant provided the letter of undertaking on the follow-up measures to be fulfilled post-authorisation on 16 December 2008.

- The CHMP opinions were forwarded in all official languages of the European Union, to the European Commission, which adopted the corresponding Decision on 25 February 2009.

2 SCIENTIFIC DISCUSSION

2.1 Introduction

Platelets play a central role in the pathogenesis of atherothrombosis and in the formation of thrombi following coronary angioplasty, with and without stent implantation. Platelets initially adhere at sites of vascular injury, atherosclerotic plaque rupture, balloon angioplasty, and stenting. Platelet activation following these interactions results in the release of ADP, thromboxane A₂, and other mediators. Released ADP promotes platelet activation via the G-protein linked P₂Y₁ and P₂Y₁₂ purinergic receptors leading to further platelet activation, aggregation, and other platelet functions, such as platelet shape change, secretion, and the development of pro-coagulant and pro-inflammatory activities.

Activated platelets are recruited to sites of coronary plaque rupture and intra-arterial stenting, thereby forming aggregates that may lead to platelet-rich thrombi, vascular occlusion, tissue ischemia, and myocardial necrosis in what is collectively known as Acute Coronary Syndrome (ACS). The term ACS is a pathophysiological continuum progressing from ischemic chest pain with sudden onset and worsening (UA), to ischemia severe enough to cause irreversible myocardial damage detected with cardiac biomarkers without persistent ST-segment elevation (NSTEMI), to total occlusion of the culprit coronary artery with persistent ST-segment elevation, resulting in myocardial necrosis and elevated biomarkers (STEMI).

ACS occurs in a diverse global population and has a significant socioeconomic impact as patients require hospitalization, rehabilitation, and often suffer subsequent ischemic events.

Acute coronary syndromes will likely remain one of the leading causes of hospitalisation worldwide due to the increasing prevalence of risk factors for coronary heart disease and the increasing size of the aged population.

Options for the initial management of ACS include pharmacotherapy alone or an early invasive strategy with PCI (with or without coronary stenting) or coronary artery bypass grafting (CABG) as guided by the results of coronary angiography. The current American College of Cardiology/American Heart Association (ACC/AHA) and European Society of Cardiology (ESC) guidelines recommend an early invasive strategy for ACS patients with intermediate to high-risk features. Pharmacotherapy includes both anticoagulant and anti-platelet drugs. The current standard of care for patients with ACS includes dual anti-platelet therapy with aspirin and thienopyridine in both the acute and chronic phases of treatment. This therapy improves outcome in patients with ACS and those undergoing percutaneous coronary intervention (PCI); the high risk of for early stent-associated thrombosis is substantially reduced by dual antiplatelet therapy. Ticlopidine and clopidogrel are the two currently approved thienopyridines. They are pro-drugs requiring in vivo metabolism to form the active metabolite that binds rapidly and irreversibly to platelet P₂Y₁₂ receptors, thus inhibiting platelet aggregation mediated by the P₂Y₁₂ receptor. Clopidogrel has largely replaced ticlopidine due to its once-daily dosing regimen, improved tolerability and lowered incidence of adverse hematological side effects.

Several potential limitations of clopidogrel therapy have been identified despite loading dose of clopidogrel. This includes marked inter-individual variability in platelet inhibition and relatively slow onset of action. An association between thrombotic complications following PCI and poor antiplatelet response to the approved standard clopidogrel dosing regimen (loading dose (LD) 300 mg and maintenance dose (MD) 75 mg) has been suggested. Further, it has been shown that "non-responsiveness" to a clopidogrel 600 mg LD is a strong predictor of stent thrombosis in patients receiving drug-eluting stents, and in addition, that residual platelet aggregation above the median is associated with a 6.7-fold increased risk of major adverse cardiac events (death, myocardial infarction and target vessel revascularisation) at 1 month follow-up in patients undergoing elective PCI.

These observations suggest the possibility that higher and more consistent levels of platelet inhibition may improve clinical outcome in patients with ACS undergoing PCI.

Prasugrel, a thienopyridine adenosine diphosphate (ADP) receptor antagonist, is an orally administered pro-drug requiring in vivo metabolism to form the active metabolite (R-138727) that

irreversibly inhibits platelet activation and aggregation mediated by the P2Y₁₂-receptor. Prasugrel has a distinct chemical structure that permits efficient conversion to its active metabolite through rapid hydrolysis by carboxylesterases and then by multiple cytochrome P450 (CYP) enzymes. Once bound, a platelet is inhibited for its remaining lifespan. After prasugrel dosing is stopped, a return to baseline levels of platelet aggregation will occur as new platelets are formed. The return to baseline typically occurs over about 7 to 10 days after treatment is stopped.

Non-clinical studies indicated that, with respect to inhibiting ex vivo platelet aggregation and in vivo thrombus formation, prasugrel was approximately 10-100-fold more potent than clopidogrel and ticlopidine, respectively. Early clinical data in healthy subjects confirmed the greater platelet inhibition and more consistent response to prasugrel compared to clopidogrel. While the active metabolites of prasugrel and clopidogrel resulted in similar levels of platelet inhibition in vitro, the amount of each active metabolite generated in vivo was quite different, with prasugrel LD (60 mg) resulting in approximately 50-fold greater exposure, on pr. Mg basis, to its active metabolite compared to clopidogrel LD of 300 mg. This observation provides a mechanistic basis for the faster, higher and more consistent inhibition of platelet aggregation (IPA) observed with prasugrel.

2.2 Quality aspects

Introduction

Efient contains prasugrel hydrochloride as active substance. Prasugrel is a member of the thienopyridine class of antiplatelet agents. Currently available thienopyridines include clopidogrel and ticlopidine. Prasugrel is an orally bioavailable prodrug metabolized to an active adenosine diphosphate (ADP) receptor antagonist, which is a potent inhibitor of platelet activation and aggregation mediated by the P2Y₁₂ ADP receptor.

Efient is an immediate release, double-arrow shaped, film-coated, debossed tablet. Tablets contain either 5 or 10 mg of prasugrel and different strengths are differentiated by size, film-coating colour and debossing. The tablets are commercially supplied in blister packaging.

Active Substance

The INN name of the active substance is prasugrel which is present in the product in the form of the hydrochloride salt. The chemical name is 5-[(1*R,S*)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate hydrochloride corresponding to the molecular formula C₂₀H₂₀FNO₃S•HCl and molecular mass of 409.90

Prasugrel hydrochloride is white to light brown crystalline solid, slightly hygroscopic and soluble to slightly soluble at pH 1-4, very slightly soluble at pH 5 and practically insoluble at pH 6-7. The pK_a value of prasugrel hydrochloride was 5.1. It shows polymorphism. It is obtained as a racemic mixture; therefore, it shows no optical rotation.

Prasugrel hydrochloride is a prodrug. In aqueous media, cleavage of the ester moiety forms the hydrolysis product, which exists as a mixture of diastereomers, and which are the precursors of the active metabolite. The hydrochloride is used because of its better hydrolytic stability and because it provides a better solubility at relevant physiological pHs.

- **Manufacture**

The synthetic route involves 3 steps where production of an intermediate, production of prasugrel free base and production of prasugrel hydrochloride consecutively takes place.

. In-process controls performed are suitable to control the reaction progress. The starting materials are considered simple molecules and satisfactory specifications were presented.

During development of the drug substance manufacturing process, quality attributes of the drug substance were evaluated with respect to the drug product manufacturing process and with respect to their impact on the critical quality attributes of the drug product. This analysis resulted in the identification of six drug substance critical quality attributes. The drug substance specification has been established to confirm that the manufacturing process reproducibly and reliably produces a drug substance that meets the critical quality attributes. Potential critical process parameters (CPPs) were identified by statistical design methods and a mechanistic understanding of the drug substance process.

All manufacturing steps are thoroughly examined and design spaces have been developed. Concerning the use of concentrated HCl, there is a potential risk that acetone is converted into diacetone alcohol and then to mesityl oxide. However, any level in the final substance is below the LOQ, which is below the limit of toxicological concern. The same synthetic route has been used to prepare all of the prasugrel hydrochloride salt used in clinical and development studies and it is the synthetic route for commercial drug substance manufacture.

- **Specification**

The drug substance specification includes tests for appearance (visual), identification (IR for prasugrel selective precipitation for Cl), assay (HPLC), impurities (HPLC), residual solvents (GC), water (Karl Fischer), Fineness (sieving) and Specific Surface Area (BET).

Results for 3 commercial scale batches were provided analyzed by the current analytical methods and against the current specifications. The results comply with the specification.

In addition, results of another numerous historical batches were provided as supportive data. However these batches were tested by analytical methodologies and against specifications that both have evolved during development.

- **Stability**

Three pilot batches (50% of full scale) were put on long-term (25°C/ 60%RH) and accelerated (40°C/ 75%RH) stability testing conditions respectively. In addition results from supporting stability studies were presented on another three earlier batches manufactured at both pilot and full scale. However the use of different equipment in the manufacture of the pilot and early batches resulted in differences in the chemical stability of the active substance and therefore the equipment yielding to more stable material was chosen. All these batches have been stored in the proposed market packaging with the exception of the supporting stability batches where desiccant was not included. 24 months of stability data were available at the long-term storage condition of 25°C/60% RH for the primary stability studies, up to 36 months for the supporting stability studies and six months data under accelerated conditions.

It was apparent from the results that generally no significant changes are seen neither at 25°C/60% RH nor at 40 °C/ 75 %RH except for an impurity which increased, but still within the limit.

The photostability of prasugrel hydrochloride in the solid state was assessed and results showed that it does not need to be protected from light in the solid state.

Finally stress testing studies have been conducted on prasugrel hydrochloride drug substance in order to gain an understanding of its degradation chemistry

The conclusion from the stress degradation, long term, and accelerated stability studies is that prasugrel hydrochloride drug substance is stable when packaged in the container closure system proposed. Prasugrel hydrochloride is susceptible to hydrolysis and therefore contact with water should be avoided. The results of these primary stability studies demonstrate that the drug substance is stable when stored at room temperature in the appropriate packaging system. The data collected to date support the proposed retest period.

Medicinal Product

- **Pharmaceutical Development**

Prasugrel hydrochloride is a prodrug. Initial clinical studies were conducted using prasugrel free base tablets. However, prasugrel hydrochloride was selected for commercial development based on the higher solubility of this salt form relative to the free base across the gastrointestinal pH range. Initial trials however exhibited undesirable degradation product formation and demonstrated that the hydrochloride salt was more susceptible to hydrolysis than the free base.

Nevertheless the use of the salt rather than the free base was selected as a result of clinical data indicating that the rate and/or extent of absorption of the free base is adversely affected if the patient takes concomitant medications, which increase gastric pH. Above pH 6, the bioavailability of prasugrel free base was substantially reduced. Based on these results, it was decided to develop the

prasugrel hydrochloride tablet formulations. Solubility determination results and permeability and metabolism information suggest that prasugrel HCl is a BCS class 2 compound.

Also, a food effect study and a study with a gastric pH modifier were conducted in humans to assess the in vivo performance of prasugrel hydrochloride or prasugrel free base tablets.

As prasugrel hydrochloride is susceptible to both hydrolytic and oxidative degradation, the formulation, manufacturing process, and packaging of tablets focused on approaches to maintain product stability.

An extensive formulation development has been conducted. Design spaces have been defined through statistically-designed and individual studies. Critical and non-critical process parameters have been defined. A finished product specification covering all normal parameters has been set up. The quality features are provided in prasugrel hydrochloride tablets using the concepts and elements of Quality by Design with risk assessment and risk mitigation in order to ensure that key product attributes were defined at an early stage.

The choice and function of the excipients in the formulation was based on the need for excipients that have the smallest possible impact on the degradation of the drug substance in formulation and the physical properties necessary for the manufacturing process.

During development, core tablet strengths ranging from 5 mg to 15 mg were developed that are qualitatively identical and quantitatively vary only in the percent w/w drug loading with concomitant adjustment of the diluent, the percentage of the other excipients in the core tablet are identical. A standard film coating is applied to produce tablets of uniform colour.

A number of studies were conducted to determine formulation robustness of the process and the formulation. Dissolution is affected by pH and decreases with increasing pH.

Clinical studies have demonstrated that tablet performance is not affected by formation of the free base over a range of 5%-70% conversion. AUC and C_{max} of the active metabolite were bioequivalent after 1 hour.

A reaction between prasugrel HCl and an excipient was observed late in the development studies during manufacture and storage. This reaction leads to a partial and irreversible formation of prasugrel free base in the tablets. Analysing the samples used for clinical phase 3 study indicated that salt-to-base formation of at least up to 70% had no clinical impact and a requirement has been included in the finished product specification.

Due to prasugrel hydrochloride susceptibility to hydrolytic and oxidative degradation a dry manufacturing process was selected. Extensive experiments have been conducted to ensure a robust manufacturing process through design spaces. This has been used to set up the process controls for the production batches. The container has been chosen to minimize humidity and to provide the necessary oxygen protection through out the shelf life of the product.

- **Adventitious Agents**

None of the excipients are animal-sourced, thus eliminating any risk of TSE contamination in the tablet formulation. The film-coating colour mixture utilizes a single animal-sourced excipient, lactose monohydrate. The source of the lactose complies with regulations to ensure patient safety.

- **Manufacture of the Product**

A dry manufacturing process is utilised for the manufacture of Efient comprising the following steps: blending, dry granulation, blending, compression, coating and drying of tablets, packaging. The manufacturing process is sufficiently described and in-process controls are adequate.

Validation data on three commercial-scale 5 mg batches and three commercial-scale 10 mg batches provided satisfactory reassurance for the reproducibility and consistency of the manufacturing process.

- **Product Specification**

The specifications of the drug product at release and shelf-life include tests for appearance (visual), identity (IR), assay (HPLC), uniformity of dosage units (Ph.Eur.), degradation products (HPLC), dye identity test (not routinely), dissolution (Ph.Eur.), tablet form conversion (XRPD).

Batch results are provided for commercial scale batches and clinical trials batches. The results comply with the specification, confirm consistency of the product and support the acceptance criteria.

- **Stability of the Product**

Stability studies have been conducted according to ICH guidances.

Three production scale batches of 5 mg tablets have been stored at 25°C/60% RH for 12 months, at 30°C/75% RH, for 12 months and at 40°C/75% RH for 6 months in the proposed market packaging.

Another three production scale batches of 10 mg tablets have been stored at 25°C/60% RH for 18 months, at 30°C/65% RH, for 18 months and at 40°C/75% RH for 6 months in the proposed market packaging.

Bulk simulator samples of both 5 and 10 mg tablets were also stored at 25°C/60% RH and at 5°C for 12 months, at 40°C/75% RH for 1 month and at -20°C for 1 month.

Additionally a supporting study for the 10 mg tablets stored in blisters was presented.

Stability results indicate that all tested parameters remain within the specification limits. Degradation products levels tend to increase but comply with the individual specification requirements at long term conditions throughout 24 months (statistically).

Photostability: A production scale batch of each strength was tested according to ICHQ1B. It is concluded from the results that no special precautions are required since the blister provides the necessary protection and the product is to be labelled to be kept in the original package.

Stress testing: A production scale batch of each strength was used for stress testing together with a placebo.. It was found that the prasugrel in the tablets degraded with exposure to heat and moisture, particularly in an ambient oxygen environment. Prasugrel does not degrade significantly with exposure to simulated sunlight.

Tablet Form Conversion: Primary stability samples were analyzed for the level of prasugrel free base after storage in both bulk and commercial packages. The conversion of prasugrel hydrochloride to prasugrel free base is primarily due to exposure to moisture. . However the present manufacturing method is shown to provide the necessary protection against moisture.

Discussion on chemical, pharmaceutical and biological aspects

The quality of Efient film-coated tablet is adequately established. Information on development, manufacture and control of the drug substance has been presented in a satisfactory manner. The quality of the active substance is considered sufficiently described and adequately supported by data. Sufficient chemical and pharmaceutical documentation relating to development, manufacture and control of the drug product has been presented. The results of tests carried out indicate satisfactory consistency and uniformity of important product quality characteristics, and these in turn lead to the conclusion that the product should have a satisfactory and uniform performance.

Stability tests indicate that the product under ICH guidelines conditions is chemically stable for the proposed shelf life.

2.3 Non-clinical aspects

Introduction

Prasugrel belongs to the thienopyridine class of prodrugs and is inactive *in vitro*. Initial studies required dosing animals with prasugrel with subsequent blood collection to look for *in vivo* activation as reflected in *ex vivo* pharmacodynamics measurements. Once the *ex vivo* evidence for the activation of prasugrel *in vivo* was established, subsequent studies addressed the potential activity of prasugrel in disease models of target indications (thrombosis). Prasugrel administration resulted in prolongation of the bleeding time as did clopidogrel and ticlopidine as it was seen in a model of haemostasis.

Consistent with differing mechanisms of action, co-administration of aspirin showed additive/synergistic interaction in studies of both thrombosis and haemostasis. Having established the *in vivo* activity of prasugrel in disease models reflecting the clinical target indication, studies were performed to characterize the activities of the active metabolite of prasugrel by *in vitro* studies.

The rationale for the non clinical development and application for marketing approval of prasugrel is considered well established. The extent and scope of the documentation provided are appropriate to characterise the non clinical profile of the product.

The following guidelines were considered: Note for guidance on safety pharmacology studies for human pharmaceuticals (CPMP/ICH/539/00), Note for Guidance on Toxicokinetics: the assessment of systemic exposure in toxicity studies (CPMP/ICH/384/95), Non-clinical guideline on drug-induced hepatotoxicity (CHMP/SWP/150115/2006), Note for Guidance on carcinogenicity: testing for carcinogenicity of pharmaceuticals (CPMP/ICH/299/95), Note for Guidance on the detection of toxicity to reproduction for medicinal products and toxicity to male fertility (CPMP/ICH/386/95), Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling (EMA/CHMP/203927/05), and Guidance on the Environmental risk assessment of medicinal products for human use (CHMP/SWP/4447/00).

The analytical method validation study in beagle dogs for the pharmacokinetics of prasugrel active metabolite was conducted in compliance with the GLP guidelines.

The safety pharmacology studies provide an evaluation of the safety pharmacology of prasugrel and meet the standards for general pharmacology studies in effect at the time of their conduct. This is considered acceptable by the CHMP. All pivotal toxicity studies were conducted in compliance with GLP regulations.

Pharmacology

- Primary pharmacodynamics

In *ex vivo* studies with rats, dogs, and cynomolgus monkeys, prasugrel demonstrated dose-dependent inhibition of ADP-induced platelet aggregation. Unless indicated otherwise, platelet function studies were performed using light transmission aggregometry (LTA), which monitors the increase in light transmission in stirred suspensions of platelets in citrated plasma (platelet-rich plasma, PRP) as they aggregate in response to activation with agonists such as ADP. ADP is a natural ligand for the target receptor (P2Y₁₂) of the thienopyridine class of oral antiplatelet agents (ticlopidine, clopidogrel, and prasugrel).

The selectivity of prasugrel for antagonism of ADP-induced platelet aggregation was demonstrated by the lesser inhibition of aggregation achieved with thrombin *vs* ADP in platelets under *ex vivo* conditions. Prasugrel's inhibitory effects were maintained after washing of the platelets, showing an irreversible platelet inhibition. Studies in rats compared prasugrel's potency with that of clopidogrel and indicated a faster onset of action, since prasugrel (1-10 mg/kg, p.o.) caused dose-dependent inhibition of platelet aggregation at 0.5 hr after dosing with an ED₅₀ value of 4.2 mg/kg, suggesting an early onset of action. In contrast, clopidogrel (10-100 mg/kg, p.o.) showed moderate effect at 0.5 hr (ED₅₀ > 100 mg/kg). The maximum effect of both prasugrel and clopidogrel were observed at 4 hr after administration; the ED₅₀ values being 1.1 mg/kg (p.o.) and 15 mg/kg (p.o.), for prasugrel and clopidogrel, respectively. The inhibitory effects of prasugrel (1- 3 mg/kg) and clopidogrel (10-30 mg/kg) were long-lasting, and these inhibitions completely disappeared at 96 hr after administration.

The *ex vivo* effects of prasugrel on platelet aggregation in male cynomolgus monkeys assessed as the ADP (10µM)-induced platelet aggregation in platelet-rich before and after oral administration of prasugrel showed that prasugrel (0.1 and 0.3 mg/kg/day) given orally once a day for 14 days inhibited platelet aggregation in a dose-dependent manner. The inhibitory effect reached a plateau on days 3 to 5, suggesting cumulative effects of prasugrel, and was maintained during the administration of prasugrel after reaching the maximal effect. The effects slowly declined after cessation of prasugrel administration. There were no significant inhibitions of platelet aggregation on the 7th day after the final dose of prasugrel (day 21). These results indicate that repeatedly administered prasugrel exhibits a potent and long-lasting antiplatelet effect.

Inhibitory effects of 14 day lasting repeated administration of prasugrel (0.03-0.3 mg/kg/day, p.o.) on platelet aggregation in the beagle dog were investigated. The ADP (8µM)-induced platelet aggregation

measurements showed inhibitory effects of prasugrel (0.1 and 0.3 mg/kg/day) reaching plateau on day 3. After cessation of administration, inhibition of platelet aggregation gradually decreased.

The *in vivo* effects of prasugrel were assessed in various non clinical pathophysiological models of thrombotic challenge:

- The arterio-venous shunt model
- The electrical injury model
- The stroke model
- The pathophysiological model of peripheral artery disease
- The bleeding time model

In the rat arterio-venous shunt model, prasugrel reduced thrombus formation in a dose-dependent manner. Similarly, prasugrel prolonged the time to occlusion and increased the patency in the electrical injury model of arterial thrombosis in a dose-dependent way. The cumulative inhibitory nature of repeat dosing with thienopyridines was demonstrated using the same model during a repeated 3 day dosing regimen. Treatment with prasugrel resulted in a dose-dependent reduction of the incidence, total area, and the number of cerebral infarcts in a model of embolic cerebral infarction in the rat, while clopidogrel had lower activity. In a model of peripheral arterial disease whereby injection of lauric acid into the rat femoral artery produces endothelial injury, platelet adhesion, and platelet aggregation, prasugrel dose-dependently inhibited progression of the lesions. Prasugrel also caused a prolongation of bleeding time in a tail transection model in rat.

Prasugrel contains a chiral centre and thus, exists as two individual enantiomers: the R-enantiomer (R-96875) and the S-enantiomer (R-96876). The platelet inhibitory effects of the individual enantiomers were evaluated following the oral administration to both, rats and monkeys, and following single oral administration of the prasugrel's individual enantiomers to beagle dogs. Additional *in vitro* studies were conducted in order to evaluate the effects on platelet aggregation.

Oral administration of R-96875 and R-96876 (both at 1 and 3 mg/kg) to rats dose-dependently inhibited platelet aggregation at 2 and 4 hr after dosing, respectively. There were no statistically significant differences in the efficacy between R-96875 and R-96876 at the same dosage. The ED₅₀ values at 4 hr after dosing were 1.4 mg/kg for R-96875 and 1.3 mg/kg for R-96876. Effects of a 3 day repeated administration of R-96875 and R-96876 (both at 0.3 mg/kg/day, p.o.) on ADP-induced platelet aggregation using platelet-rich plasma were also examined in cynomolgus monkeys. The wash-out periods between the two treatments was considered acceptable. Both isomers caused inhibition of platelet aggregation on day 3, and this effect was almost equal between the groups. There were no statistically significant differences in the efficacy between R-96875 and R-96876 at any point. These results indicate that oral administrations of optical isomers of prasugrel, R-96875 and R-96876, exert a similar extent of *ex vivo* effect on platelet aggregation in rats and in cynomolgus monkeys.

The active metabolite R-138727 has two chiral centres, resulting in four enantiomers. Metabolite R-138727 has potent and selective P2Y₁₂ antagonistic activity. The two most potent enantiomers of R-138727, the R-125690 and R-125689, are about 100- and 10-fold more potent than enantiomers R-125687 and R-125688, respectively. The two most potent enantiomers comprised the majority of the circulating R-138727 in rats and humans.

In pharmacodynamic mechanistic studies, the active metabolite of prasugrel, R-99224, affected P2Y₁₂-specific biomarkers, including alpha granule release, fibrinogen binding, and restoration of ADP-induced reduction of PGE₁-induced elevation cAMP. In contrast, P2Y₁ biomarkers (platelet shape change, Ca²⁺ mobilisation) were unaffected by pre-incubation of platelets with prasugrel's active metabolite. This confirms that the inhibition of the platelet aggregation by prasugrel is mediated by P2Y₁₂ receptors. The pharmacological effects are most probably dependant on the production of the active metabolites of prasugrel.

Inhibitory effects of orally administered optical isomers of prasugrel on platelet aggregation investigated in beagle dogs was measured as the platelet aggregation induced by ADP (8 µM) at 2 and 4 hr post dosing. There were no significant differences in baseline values of platelet aggregation

among all groups. In the control group, there were no obvious changes in aggregation after vehicle administration. In contrast, each isomer (0.1-1 mg/kg, p.o.) inhibited platelet aggregation in a dose-dependent manner. There were no statistically significant differences in platelet aggregation at 2 and 4 hr post dose between the two isomers at corresponding doses. In addition, ED₅₀ values for the two isomers were similar at 2 and 4 hr post dose. These results show that an oral administration of the optical isomers of prasugrel produces anti-platelet effects of similar potency in beagle dogs. This pharmacodynamic study supports the use of racemic prasugrel, since all four enantiomers are formed as was shown in a pharmacokinetic study in dogs.

In general, the *ex vivo* studies with rats, dogs, and cynomolgus monkeys demonstrated dose-dependent inhibition of ADP-induced platelet aggregation by prasugrel. Studies in rats also demonstrated prasugrel's potency compared to clopidogrel and suggested a faster onset of action. The selectivity of prasugrel antagonism of ADP-induced platelet aggregation was demonstrated by the lesser inhibition of aggregation achieved with thrombin vs ADP in platelets *ex vivo*. The inhibitory effect was maintained after washing of the platelets, showing an irreversible platelet inhibition.

- Secondary pharmacodynamics

No secondary pharmacodynamic studies were made on binding and activity to other proteins than P2Y₁₂ and P2Y₁ and the secondary pharmacology data were derived from the results of the safety pharmacology studies. The effects observed in these *in vitro* safety studies occur at a concentration at least more than 10-fold higher than the maximum therapeutic concentration observed in humans. Both, the non clinical *in vivo* studies and clinical studies, did not provide any evidence for unexpected secondary pharmacodynamic effects of prasugrel. Thus, further studies are deemed unnecessary. Nevertheless, the results of a screening of prasugrel and its metabolite M1 in a standard battery of receptor binding assays were requested by the CHMP. Thus, M1 and prasugrel were tested in a battery of receptor binding assays. Neither prasugrel nor M1 showed affinity for the tested receptor at concentrations up to 10 µM.

- Safety pharmacology programme

Assessments of *in vivo* activity of prasugrel included evaluation of cardiovascular, central nervous system (CNS), respiratory, renal, and gastrointestinal (GI) functioning in rodents or dogs.

Effects on the GI and CNS occurred at high doses of prasugrel. At an oral dose of 100 mg/kg, prasugrel produced a significant decrease in paradoxical sleep in rats, without altering the total percentage of time spent sleeping. Increased sensitivity to touch was observed in rats at the 300 mg/kg oral dose. Other CNS endpoints, including body temperature, clinical observations, precipitated seizure thresholds, spontaneous activity, and thiopental-induced sleep times, were not altered following administration of single oral doses of prasugrel up to 300 mg/kg. Examination of the effects on autonomic nervous system and smooth muscle showed that prasugrel inhibited spontaneous movement of isolated rabbit ileum at 1x10⁻⁴ g/ml and inhibited the amplitude and increased frequency of spontaneous motility of isolated pregnant rat uterus. Prasugrel at 1x10⁻⁵ g/ml significantly inhibited acetylcholine-, histamine- and serotonin induced contractions in isolated guinea pig ileum.

The potential for prasugrel to inhibit cardiac repolarisation was evaluated by examining the effect of three prasugrel metabolites on potassium currents in hERG-transfected cells. The metabolites R-138727 and R-106583 were evaluated because these are the active and the most abundant inactive human metabolites, respectively, and R-95913 was evaluated because it is the intermediary step between prasugrel and the active metabolite. No significant effects on the potassium currents in hERG-transfected CHO-K1 cells were observed at up to the highest concentrations tested (30 µM for R106583 and R138727; 15 µM for R-95913) which were greater than approximately 485 times the expected free C_{max} values of the three metabolites following a clinical loading dose of 60 mg prasugrel. Therefore, the hERG data for prasugrel metabolites do not suggest a potential impact of prasugrel on cardiac repolarisation due to inhibition of potassium currents. Prasugrel (30 and 100 mg/kg, ID) showed no major effects on heart rate, blood pressure, respiration rate, carotid blood flow, or pressure response to acetylcholine, norepinephrine or bilateral carotid occlusion in the anaesthetised dogs. No effects on QT interval were observed in quantitative electrocardiograms evaluated in the 3 and 9

month repeat dose studies in dogs at doses approximately nine times the 60 mg clinical loading dose calculated as mg/m².

Prasugrel produced a decreased gastric acid content and gastric volume at 100 mg/kg in rats. Furthermore, prasugrel decreased gastric emptying in mice when given for 3 days at the dose of 300 mg/kg. However, the doses at which these effects occurred were ≥ 14 times the 60 mg clinical loading dose calculated as mg/m². Prasugrel (10-100 mg/kg, p.o.) had no effects on urinary volume, excretion of electrolytes or osmotic pressure in rats.

- **Pharmacodynamic drug interactions**

Thienopyridine antiplatelet agents are commonly used in combination with aspirin as “dual antiplatelet therapy”. The use of the combination is based on the alternative receptor/signalling pathways that each of these agents inhibits and the additive, or synergistic, platelet inhibitory effects that results from co-administration. Pharmacodynamic studies were conducted with the combination of prasugrel/aspirin. An additional study involved co-administration of other drugs, in which the comparisons of the pharmacokinetics and pharmacodynamics of prasugrel base and hydrochloride salt were made in the presence of the proton pump inhibitor lansoprazole.

The additive activity of prasugrel and aspirin has been demonstrated in several studies of platelet aggregation (*ex vivo*) in rats and dogs, thrombus formation (*in vivo*) in rats, and bleeding time in rats. Consistent with these findings, *in vitro* studies with blood from human volunteers demonstrated that a combination of R-138727 and aspirin has additive effects on collagen-induced platelet aggregation.

The antiplatelet effects of two tablet formulations of prasugrel, the free base tablet and hydrochloride salt tablet, were compared in beagle dogs pretreated with lansoprazole, a proton pump inhibitor. Plasma concentrations of prasugrel metabolites at 1 hr post dosing were not significantly different from those of the free base tablet and hydrochloride salt tablet given to dogs. These results suggest that the free base tablet and hydrochloride salt tablet have similar antiplatelet potency in lansoprazole-treated dogs.

Pharmacokinetics

Absorption, distribution, metabolism, and excretion profile of prasugrel was investigated in mice, rats, and dogs, which are also the species used in the toxicological evaluation of the compound. Analytical methodology evolved adequately. In initial pharmacokinetic and absorption studies, some inactive metabolites of prasugrel were measured and their pharmacokinetic parameters used as indicators of the absorption and metabolism of prasugrel. A number of new metabolites were quantified and a method for determination of prasugrel's active metabolite concentrations in plasma was ultimately developed. Most studies were conducted following oral administration, the intended clinical route of administration.

Prasugrel is rapidly absorbed in all species including humans; T_{max} of the active metabolite R-138727 is less than 1 hour. Prasugrel itself was not detected in plasma after oral administration. The decline of prasugrel related radioactivity was biphasic in rats and dogs. The radioactivity terminal elimination half-life seemed to be similar in mice and rats, approximately 24 h, but it is considerably longer in dogs, approximately 3 days. In humans, the average terminal elimination half-life of the active metabolite R-138727 was approximately 7 hours. Approximately 21% of a [¹⁴C]-prasugrel dose is excreted in human faeces within 48 hours. The pharmacokinetic studies have only been conducted in male animals. However, no apparent sex differences were observed during the repeat-dose toxicity studies. Following single oral doses of prasugrel base or prasugrel hydrochloride, the exposure to prasugrel metabolites was evaluated in the mouse, rat, and dog. Exposure parameters to prasugrel metabolites were higher for prasugrel hydrochloride compared with prasugrel base at doses of ≥ 500 mg/kg in the rat and at 100 mg/kg in the dog. Tissue distribution of radioactivity related to prasugrel was studied in rats following single and repeated oral administration. Radioactivity was widely and rapidly distributed throughout the body. The radioactivity concentration was highest in most tissues involved in the absorption and elimination of the compound and its metabolites, i.e., stomach, intestines, liver, kidney and urinary bladder. Prasugrel distributed to the bone marrow of rats with a

tissue-to-plasma ratio of less than 0.5. Following repeated daily dosing, accumulation consistent with the elimination half-life of prasugrel was observed in most organs. After a single oral dose of 5 mg/kg ^{14}C -prasugrel to rats on Day 13 of pregnancy, the fetal concentration of prasugrel radioactivity was 0.27 times that in maternal blood 1 hour after administration and declined thereafter, suggesting low placental transfer of prasugrel or its metabolites. Due to instability of the active metabolites R-138727 in plasma, the protein binding was only investigated in human serum albumin, where the metabolite was highly bound by 98% and the species differences in protein binding of the active metabolites R-138727 were not assessed. The protein binding of the inactive metabolites R-100932, R-106583 and R-95913 was similar in rats, dogs and humans (>80%) while the protein binding of the inactive metabolite R-119251 was significantly lower in dogs (26-36%) as compared to rats (71-77%) and humans (76-77%). Prasugrel was extensively metabolised in all species. A total of eighteen metabolites were identified in human plasma. Based on a mean radioactivity above 10%, the following major metabolites could be identified: diastereomers of M1, M2 (R-95913) and M5 (R-106583). The metabolites of prasugrel found in human plasma, urine and faeces were also detected in mouse, rat and dog; the only exception being M16, which was only identified in the mouse. M16 is M10 conjugated to glucuronic acid and M10 was found in all species. Furthermore, the extent of formation of a given metabolite varied significantly by species. Metabolite M1 was formed in large amounts in humans and was detected in animal plasma, but quantification was not conducted in animals due to co-eluting of the radioactive peaks. Metabolites M2, M5, M7 and M14 were also formed in larger amounts in humans as compared to the animal species.

In dogs, the hydrolysis of prasugrel led to a formation of essentially equal amounts of the four enantiomers of R-95913. All enantiomers of the active metabolite R-138727 were generated from R-95913. The R-125690 and R-125689 enantiomers accounted for approximately 50-64% of the R-138727 in dog plasma and >99% in rat plasma. The CHMP also inquired about levels in mice and rabbits. It was shown that all tested animal species were exposed to the most potent of enantiomers of R-138727, R-125690 and R-125689, at concentrations significantly higher than those observed in humans and thus, adequate margins of safety could be assured. Also, all animal species were exposed to the least potent of R-138727 enantiomers at concentrations higher than those in humans, with the exception of the rat as R-125687 and R-125688 concentrations could not be quantified.

Considering that isomers can have different or even antagonistic effects towards the same receptor system, these opposite effects might occur in species are capable of forming all four enantiomers of R-138727. Nevertheless, in the course of several studies of the antagonistic profile of the enantiomers using light transmission aggregometry, no evidence of agonistic activity was noted between R-125690 and R-125689 isomers of R-138727.

When administered at high doses (≥ 100 mg/kg) to rats, prasugrel induced CYP450 enzymes (CYP2B and CYP3A2) and phase II metabolizing enzymes UDP-glucuronosyltransferase and glutathione-S-transferase, however, based on the *in vitro* non clinical study with human hepatocytes, this induction is not observed in humans. Furthermore, the AUC for each measured metabolite decreased after multiple dosing compared with the values obtained after the first dose in mice at ≥ 100 mg/kg/day, in rats at 100 and 300 mg/kg/day, and in dogs at 20 mg/kg/day (after 20 weeks of dosing and beyond). However, the exposure data in dogs administered prasugrel at 20 mg/kg for one month were essentially unchanged. In the nine month study with dogs, the AUC data for two of the metabolites R-100932 and R-106583 decreased after 20 weeks of dosing, while the AUC values of the other metabolite, R-95913 were higher in dogs dosed with prasugrel at 20 mg/kg. Thus, the data show some auto-induction of prasugrel's metabolism at the 20-mg/kg dose in dogs. Since induction was not observed either *in vitro* or *in vivo* in humans and the non-clinical data suggest that induction of CYP3A4 due to administration of prasugrel is unlikely at clinically relevant plasma concentrations, this is not considered a major issue.

In mice, 90% of the dose was excreted during the first 24 hours post dosing mainly *via* the urinary elimination route. In rats and dogs, the majority of the radioactivity (>90%) was excreted within the first 72 hours of dosing in faeces presumably *via* bile. Approximately 20% of the dose was excreted *via* urine. Radioactivity related to prasugrel was also detected in milk of lactating rats at

concentrations up to approximately five times higher than the plasma level. However, the radioactivity from milk ($T_{1/2}=9.5$ h) was eliminated more rapidly than that from the plasma ($T_{1/2}$ approximately 24 h).

No pharmacokinetic drug interactions studies were conducted in animals and this was justified with the sufficient evaluation of pharmacokinetic drug interactions in a clinical setting.

Toxicology

The toxicological and toxicokinetic profile of prasugrel was investigated in a comprehensive programme, including studies on systemic toxicity after single and repeat dose administration, reproductive toxicity studies, genotoxicity studies as well as studies addressing specific issues, such as antigenicity, phototoxicity, toxicity of impurities, dermal and ocular irritation.

Prasugrel base was used during the major part of the toxicology program. However, a change was made to the hydrochloride salt of prasugrel later in development. Repeat dose studies comparing the base and the hydrochloride salt of prasugrel were conducted in mice with two week duration and rats and dogs with one month duration. A single dose comparison study was conducted in rats.

- **Single dose toxicity**

Single dose toxicity studies following the oral administration were conducted in rats and mice at doses up to 2000 mg/kg. Clinical observations in female rats given 2000 mg/kg included some non-specific signs of irregular respiration, reduced locomotor activity, ptosis, lacrimation, and staggering gait. In a comparison single dose rat study of prasugrel base vs prasugrel hydrochloride, no deaths occurred at doses of prasugrel base up to 2000 mg/kg, while 3 out of 5 males and 4 out of 5 females administered 2000 mg/kg prasugrel hydrochloride died. Systemic exposure to prasugrel metabolites in the prasugrel hydrochloride group was 1.2 to 3.5 times higher than that of the prasugrel base group and this difference is believed to account for the difference in mortality. In an escalating dose study in beagle dogs, platelet aggregation was inhibited, consistent with the pharmacological action of the compound. Emesis was observed after administration at doses ≥ 300 mg/kg, and serum ALP was increased following the 2000 mg/kg dose. Slight hepatocellular atrophy and ground glass appearance of hepatocellular cytoplasm were also observed in these dogs. Data from mice, rat and dog showed that prasugrel has low acute toxicity.

- **Repeat dose toxicity (with toxicokinetics)**

Repeat dose studies of up to three, six, and nine months in duration were conducted with prasugrel administered orally to mice, rats, and dogs. In most of these studies prasugrel base was used as the tested compound. Bridging studies comparing the toxicity of prasugrel base and prasugrel hydrochloride were conducted in each species.

Mortality, decreased body weight, and anaemia were observed in mice at repeated administration of a dose of 1000 mg/kg prasugrel. Anaemia was attributed to subclinical blood loss rather than to haematopoietic suppression, since an increase in the reticulocyte ratio was also observed, and there were no histologic effects on bone marrow. Liver was the primary target organ and increased liver weight and hypertrophy of centrilobular hepatocytes most likely due to induction of drug metabolising enzymes were observed. In the two week study, increased ALT and AST activity and the single cell necrosis indicated toxic effects on liver at a 2000 mg/kg dose of prasugrel, which was also lethal. The maximum tolerated dose (MTD) of prasugrel in mice was considered to be 300 mg/kg. Similar effects were observed in a fourteen day bridging study conducted in mice to compare the toxicity of prasugrel base and prasugrel hydrochloride. Some effects, e.g. the decreased erythrocytic parameters and liver histopathology findings, were more apparent in the prasugrel hydrochloride group.

No animals died during the studies in which rats were administered 0, 10, 30, 100, and 300 mg/kg of prasugrel orally for 3 months. Body weights decreased relative to control by 10% and 6% for males and females, respectively, in the 300 mg/kg group. Platelet counts increased in males given ≥ 100 mg/kg and in females given 300 mg/kg. Prothrombin times in males and activated partial thromboplastin times were prolonged in rats receiving ≥ 100 mg/kg. Evidence of enzyme induction included increased liver weight, hypertrophy, and acidophilic cytoplasm of hepatocytes, in male rats

given ≥ 100 mg/kg and female rats given 300 mg/kg. The enzyme induction effects and alterations in coagulation parameters were considered compensatory and pharmacologic in nature and thus not adverse.

Administration of similar doses of prasugrel orally for 6 months did not result in any deaths and similar blood effects were observed at higher doses. Clinical chemistry effects included decreased total cholesterol in males of the 300 mg/kg group, decreased triglycerides in males of the 100 mg/kg and slight decrease in potassium and chloride in females of the 300 mg/kg group. These changes were attributed to decreased food consumption. Increased total bilirubin, total protein and β -globulin and albumin were thought to be caused by the acceleration of protein synthesis in the liver accompanying induction of drug metabolizing enzymes. An increase in calcium was observed in both sexes given doses ≥ 100 mg/kg and was considered to be due to the increase in serum protein and the consequent increase in protein bound calcium. Liver weight increase was noted. Histopathological examination revealed hypertrophy of the hepatocytes and are consistent with the occurrence of enzyme induction. Other changes included decreased thymus weight in females of the 100 and 300 mg/kg groups, decreased prostate weight in the 100 and 300 mg/kg groups, and decreased uterine weight in the 300 mg/kg group. These were all slight changes without accompanying histopathological changes. The NOAEL of prasugrel in this study was 30 mg/kg.

Prasugrel base and prasugrel hydrochloride were administered daily for 28 days to rats to examine their differences in toxicity. Prasugrel hydrochloride was administered at dose levels of 0, 30, 100, and 300 mg/kg, and prasugrel base was administered at 300 mg/kg. Decreased body weight gain associated with decreased food consumption was recorded in females at 100 mg/kg and in males and females at 300 mg/kg with prasugrel hydrochloride. Administration of prasugrel hydrochloride was associated with a tendency toward decreased erythrocyte parameters in males and females at 300 mg/kg and an increase in reticulocyte percentage in females at 300 mg/kg. Platelet count, activated partial thromboplastin time, and fibrinogen were also increased. Anomalous levels of triglycerides, glucose, potassium, chloride, calcium, total protein, albumin, α 2-globulin and β -globulin were observed. These findings were comparable at 300 mg/kg between prasugrel hydrochloride and prasugrel base. The observed increase in liver weight, thought to be caused by an induction of drug metabolizing enzymes, was observed in males at 30 mg/kg and in males and females at 100 mg/kg and above in the prasugrel hydrochloride group and at 300 mg/kg in the prasugrel base group. Macroscopic examination revealed dark discoloration of the liver in males and females at 300 mg/kg for both compounds, and histopathological examination revealed hypertrophy of hepatocytes at each dose level for both compounds. The quantitative differences in exposure parameters and toxicological findings between prasugrel base and hydrochloride were discussed by the CHMP, especially in terms of the choice of the compound for the long term toxicological studies. It was, however, justified that the observed differences in animals treated with prasugrel hydrochloride or prasugrel base were not indicative of qualitative differences in toxicologic responses. The comparability of the pharmacokinetic and toxicity profiles between the base and the salt in bridging studies up to one month in duration in mice, rats, and dogs supported the appropriateness of using the salt for long term toxicology studies.

Beagle dogs were administered 0, 0.8, 4, or 20 mg/kg of prasugrel orally for 3 and 9 months. In animals receiving 4 mg/kg or more, hypertrophy of hepatocytes accompanied by the ground glass appearance of cytoplasm was observed. Animals receiving 20 mg/kg showed increased alkaline phosphatase activities and electron microscopic examination revealed a slight proliferation of the smooth endoplasmic reticulum in hepatocytes. These changes were considered to be due to activation of drug metabolism enzymes induced by administration of prasugrel. Decreased total cholesterol levels occurred in animals receiving 20 mg/kg.

An oral toxicity study to compare the toxicities of prasugrel base and prasugrel hydrochloride was conducted in which the compounds were administered orally once daily for 28 days. There were no compound related clinical signs or effects on body weight, food consumption, ophthalmology, electrocardiography, urinalysis, haematology, or gross pathology. Elevation of ALP activity occurred in males and females of the groups at 100 mg/kg prasugrel hydrochloride and 100 mg/kg prasugrel base. The increases in alkaline phosphatase levels occurred earlier and were more pronounced in the female dogs treated with 100 mg/kg prasugrel hydrochloride than in female dogs given 100 mg/kg

prasugrel base. The histopathological examination revealed lamellar inclusion bodies in the hepatocellular cytoplasm after administration of prasugrel hydrochloride. Hypertrophy of hepatocytes was observed with both compounds and was attributed to the induction of drug metabolizing enzymes. Slight hypertrophy of the thyroid follicular epithelia was observed in a male dog given 100 mg/kg prasugrel base. The changes observed in the thyroids were secondary to the accelerated metabolism of thyroid hormones due to elevated hepatic drug metabolizing enzymes.

The histopathological liver alterations and the serum hepatic enzymes changes were observed continuously throughout the repeat dose toxicity studies in mice, rats and dogs. The CHMP's concern regarding these findings, their relevance for prediction of human hepatotoxicity, especially considering that induction of CYP450 is not observed in humans, and the overall potential hepatotoxicity of thienopyridines was appropriately addressed. Despite the lack of evidence for hepatotoxicity, hepatotoxicity is identified as a precautionary approach as a Potential Risk in the Risk Management Plan (RMP) and is subject to a range of surveillance activities.

- **Genotoxicity**

Prasugrel did not exhibit genotoxic properties when tested in a battery of standard *in vitro* (Ames and chromosome aberration) and *in vivo* (mouse micronucleus) assays. However, the CHMP requested the information concerning the purity of the tested batches and specifically, the impurity levels in the batches with regards to the genotoxicity tests. In response, the impurity level in the batches used for the pivotal toxicity studies were characterised based on an analysis of the actual amount of the impurities in the administered doses at the NOAEL. Sufficient levels have been achieved. In case of MFTP and PFTP, the level of impurities in the lots used for the *in vitro* genotoxicity studies are regarded as sufficient for qualification at the proposed specifications (0.20% and 0.15%, respectively). Although the proposed specification for OHTP (0.20%) cannot be deemed qualified by the *in vitro* genotoxicity studies, the margins of safety for the *in vivo* micronucleus study are significantly high to qualify the proposed specification for OHTP (>57 based on mg/m² and a prasugrel salt vs base exposure ratio of 3.5). Thus, OHTP, MFTP, and PFTP are considered qualified at the proposed specification.

- **Carcinogenicity**

Studies conducted over 24 months with mice at doses up to 300 mg/kg and rats with doses up to 100 mg/kg aimed at the assessment of the carcinogenic potential of prasugrel. When treated with prasugrel hydrochloride, mice developed adenomas of the liver, but not carcinomas. In view of the lack of genotoxicity, the increase in mice tumours was assumed to be caused by the adaptive enzyme induction response. The mice are prone to developing tumours under such circumstances and the mechanism is unlikely to be relevant for humans. Furthermore, hepatocellular hypertrophy, thought to be the result of microsomal enzyme induction, but no tumour induction was observed in the rat study. The increase in liver tumours in mice administered prasugrel is not considered to be a relevant human risk and this is adequately reflected in the proposed prescribing information.

- **Reproduction Toxicity**

Fertility, early embryonic development and peri- and postnatal toxicity were assessed in studies with rats and embryo-fœtal development in studies in rats and rabbits. In rats prasugrel did not exhibit toxicity on fertility and early embryonic development. In rabbits and rats prasugrel did not show signs of embryo-fœtal toxicity. Prenatal and postnatal development, including maternal function in rats was not affected by exposure to prasugrel. The SPC adequately reflects these findings. The CHMP noted a reduction in mean adrenal gland, seminal vesicle/prostate gland, and combined epididymis weights at prasugrel doses of 300 mg/kg/day in the fertility rat study. There were no treatment-related histopathologic changes in these tissues in the 3- and 6-month rat studies and no effects in the dogs, except one early two week pilot study, in which atrophy of seminiferous epithelium in testes with slight-to-moderate nature was observed at high doses. This observation in dogs was comparable with the historic controls and did not appear in rats. Further evaluation of the data confirmed there were no effects on fertility, sperm count and sperm motility in rats. Overall, no reproductive risk could be concluded.

- Toxicokinetic data

Toxicokinetic data were collected from repeated dose studies in mice, rats and beagle dogs. Safety margins based on plasma drug exposures were determined for the active metabolite R-138727 and for R-106583 in the relevant studies. In addition, safety margins based on administered dose/body surface area have also been determined (please see *Pharmacokinetics*).

- Local tolerance

No study on local tolerance was performed. This is considered acceptable since prasugrel is administered orally. However, exposure of the skin or the eyes to prasugrel may occur during the manufacturing process. Two irritation tests were conducted in rabbits. In the hazard evaluation studies conducted in New Zealand white rabbits, prasugrel was a mild ocular irritant and its administration to the conjunctival sac of rabbits resulted in iritis and conjunctivitis, which resolved within 24 hours and seven days after the treatment, respectively. Prasugrel did not cause dermal irritation following a single application of 1000 mg/kg to the skin of rabbits.

- Other toxicity studies

Antigenicity

Prasugrel was tested for antigenicity in mice and guinea pig. Based on the results obtained from these tests, prasugrel is not expected to be antigenic.

Immunotoxicity

No specific tests for immunotoxicity were conducted and this was justified by the results of standard toxicity tests or based on pharmacologic properties of the compounds. It was argued that the available clinical safety data did not reveal any prasugrel related hypersensitivity reactions or suggest any increase in infection in the prasugrel vs clopidogrel treatment groups. There is no direct link between prasugrel and allergic reactions, but due to the fact that other thienopyridines have been associated with allergic reactions, these have been identified as potential risks in the RMP and are subject to a range of surveillance activities.

Phototoxicity

Distribution studies showed that prasugrel metabolites are distributed to the skin and eye ball in levels of 1/10 of the plasma concentration after single exposure, with some potential to accumulate after repeated dosing. The phototoxic potentials of R-138727 and R-106583 were evaluated *in vitro* examining the uptake of Neutral Red in the presence or absence of light using Balb/c 3T3 cells of mouse fibroblast cell line in the range of 290-700 nm. For R-138727, the Photo Irritation Factor (PIF) was below 2 (i.e. non-phototoxic). For R-106583, the PIF was not determined because the cell survival was >50%, with or without irradiation and indicated no remarkable cytotoxicity up to the maximum concentration of 1000 µg/mL. Nevertheless, R-138727 was determined as “probably phototoxic” in a second study employing the same dose range and experimental design, with a PIF of >2 (i.e., PIF 4.31). R-106583 was negative. According to the Note for guidance on phototoxicity testing (CPMP/SWP/398/01) it was not shown that prasugrel and/or its metabolites are not phototoxic and the CHMP raised a question on this issue. In response, it was shown that other non-clinical and clinical data indicate that evidence of the phototoxic potential of prasugrel is weak and of questionable clinical relevance. Nevertheless, phototoxicity was included as a potential risk in the RMP. The lack of photoallergy and photogenotoxicity is acceptable in light of the weak evidence of the phototoxic potential.

Studies on impurities

The potential toxicities of most of the prasugrel impurities were evaluated as part of the non clinical toxicology studies. All impurities above 0.15 % were qualified either by separate genotoxicity studies and a 14-day repeat dose study or toxicological studies. Based on the studies, the overall specifications for impurities CATP and diketone were considered justified from a toxicological perspective.

Ecotoxicity/environmental risk assessment

Environmental chemistry, fate and effects data were collected for prasugrel as recommended in the Guideline for environmental risk assessment of medicinal products for human use

(CHMP/SWP/4447/00). The Phase I estimate of maximum exposure to all prasugrel residue in surface water predicted an exposure above the 0.01 µg/L and thus, a complete risk assessment (Phase II Tier A) according to the current guideline has been conducted. No likely risk has been identified with regard to aquatic organisms in either ground water or surface water, neither for sediment dwelling organisms.

2.4 Clinical aspects

Introduction

This full application concerns centralised procedure in accordance with the Regulation (EC) No 726/2004, Article 3(2)(a). It is submitted in accordance with Article 8(3) in Directive 2001/83/EC for a new active substance. Conditional approval, an approval under exceptional circumstances or an accelerated review are not requested

Prasugrel is an inhibitor of platelet activation and aggregation through the irreversible binding of its active metabolite to the P2Y₁₂ class of ADP receptors on platelets. Since platelets participate in the initiation and/or evolution of thrombotic complications of atherosclerotic disease, inhibition of platelet function can result in the reduction of the rate of cardiovascular events such as death, myocardial infarction, or stroke.

The approved indication is:

EFIENT, co-administered with acetylsalicylic acid (ASA), is indicated for the prevention of atherothrombotic events in patients with acute coronary syndrome (i.e. unstable angina, non-ST segment elevation myocardial infarction [UA/NSTEMI] or ST segment elevation myocardial infarction [STEMI]) undergoing primary or delayed percutaneous coronary intervention (PCI).

Scientific advice for the product was requested from the CHMP in 2004. The given advice concerned, among others, the population included in the clinical development programme and the number of studies to be conducted, choice of a comparator and clinical endpoints in the clinical studies, use of aspirin as co-therapy or monitoring of safety of patients. It is claimed that the relevant scientific guidelines were followed.

There is no paediatric development programme. According to the European legislation valid at the time of submission, there was no need to submit a paediatric investigation plan before July 2008.

At the time of submission, the prasugrel clinical development program consisted of 46 completed placebo-controlled or active-comparator (clopidogrel) controlled studies. In the majority of studies, subjects were randomly assigned, in an open-label or blinded fashion, to treatment using either parallel or crossover designs. Across all studies, 8656 subjects received at least one dose of prasugrel.

Summary of the key studies in the prasugrel clinical development program.

Study Alias	Study Type	Subjects (N)	Overall Conclusions
H7T-EW-TAAA, H7T-EW-TAAE, H7T-EW-TAAJ	Phase 1 Dose Ranging (single dose, multiple dose regimens) +/- aspirin Doses from 5 mg - 60 mg; daily multiple doses of 5 - 15 mg for up to 21 days	Healthy TAAA (42) TAAE (45) TAAJ (68)	Higher, faster, and more consistent IPA versus 300-/75-mg LD/MD clopidogrel
H7T-EW-TAAD	Phase 1b Dose Ranging (multiple LD/MD regimens) 28-day duration	Stable atherosclerosis (101)	Higher, faster, and more consistent IPA versus 300-/75-mg LD/MD clopidogrel
H7T-MC-TAAH	Phase 2 Dose Ranging Safety (multiple LD/MD regimens) 30-day duration	Elective and urgent PCI (905)	60-/10-mg LD/MD prasugrel showed comparable TIMI Major + Minor bleeding to 300-/75-mg LD/MD clopidogrel, trend towards decreased 30-day MACE
H7T-MC-TABR	Phase 1b Comparative PK/PD (60-/10-mg LD/MD prasugrel vs 600-/75-mg LD/MD clopidogrel regimens) 28-day duration	Stable CAD (110)	More rapid onset of higher and less variable IPA versus 600-/75-mg LD/MD clopidogrel
H7T-MC-TABL	Phase 2 Comparative PD (60-/10-mg LD/MD prasugrel vs 600-/150-mg LD/MD clopidogrel regimens). 30-day duration	Elective PCI (201)	More rapid onset of higher IPA versus 600-/150-mg LD/MD clopidogrel
H7T-MC-TAAL	Phase 3 Pivotal Study (60-/10-mg LD/MD prasugrel vs 300-/75-mg LD/MD clopidogrel regimens) with aspirin Maximum duration 15 months	PCI in ACS (13608)	Superior efficacy for 60-/10-mg LD/MD prasugrel vs 300-/75-mg LD/MD clopidogrel regimens with higher risk of bleeding

Abbreviations: ACS = acute coronary syndrome; CAD = coronary artery disease; IPA = inhibition of platelet aggregation; LD = loading dose; MACE = major adverse cardiovascular events; MD = maintenance dose; N = number randomly assigned to prasugrel and/or clopidogrel; PCI = percutaneous coronary intervention; PD = pharmacodynamic; PK = pharmacokinetic; PK/PD = pharmacokinetic/pharmacodynamic; TIMI = Thrombolysis In Myocardial Infarction. [[HG, Source Module 5.2.6]]

GCP

As claimed by the applicant, clinical trials were performed in accordance with GCP. A statement to the effect that clinical trials conducted outside the community were carried out in accordance with the ethical standards of Directive 2001/20/EC was also provided. The assessment of the clinical data did not raise concerns about their compliance with GCP. No inspection was requested.

Pharmacokinetics

Prasugrel is administered as a racemic prodrug that is metabolized *in vivo* to the active moiety, R-138727, which irreversibly binds to the platelet P2Y₁₂ receptor and blocks activation and aggregation induced by the P2Y₁₂ agonist adenosine diphosphate (ADP). The R-138727 metabolite is formed very rapidly during first-pass metabolism.

The pharmacokinetics of prasugrel's active metabolite (R-138727) in healthy subjects was assessed in various clinical pharmacology studies by conventional non-compartmental methods and by population analysis. A meta-analysis of non-compartmental pharmacokinetics estimates from 16 phase 1 studies consolidated exposure estimates from 506 healthy male and female subjects and evaluated the effect of specific subject factors on exposure to the active metabolite.

Formulation development

Prasugrel development began with prasugrel base, which was used in the earlier studies in healthy subjects and subjects with stable atherosclerosis. Decision to switch to prasugrel hydrochloride was made after study TAAC showed that the C_{max} and AUC of prasugrel's inactive metabolites were greatly reduced when prasugrel base was given to healthy subjects whose gastric pH was >6 at the time of dosing. This was believed to be of a potential consideration for patients taking concomitant treatment with proton pump inhibitors (PPIs) or H_2 -receptor antagonists, which also raise gastric pH. Because the solubility of prasugrel hydrochloride is higher than that of prasugrel base at higher pH, switching from the base to the hydrochloride salt might lessen the impact of elevated gastric pH in patients taking PPIs and H_2 -receptor antagonists. Formulation strategy for the hydrochloride salt of prasugrel focused on developing an immediate-release tablet for oral administration. Initially, a 10-mg tablet was developed, which is to be used for both, the 60-mg loading dose (LD) and the daily 10-mg maintenance dose (MD). Later a 5-mg tablet was developed to provide increased dosing flexibility. The proposed commercial 10-mg tablet formulation was used in the pivotal, phase 3 study TAAL, and thus, no bioequivalence study was performed.

- **Absorption**

Prasugrel is rapidly absorbed after oral administration and is not detected in plasma. However, prasugrel's active metabolite (R-138727) appears in plasma rapidly after the oral dosing, reaching a peak concentration (C_{max}) in about 30 minutes and declining bi-phasically with a terminal half life of approximately 7.4 hours. The average C_{max} of active metabolite is 475 ng/ml after a 60-mg LD and 70 ng/mL during 10-mg MD. The time to reach the maximum plasma concentration (t_{max}) is at a median of 0.5 hours. It was estimated that approximately 79% of a prasugrel dose is absorbed. The between-subject and within-subject variability is 27.6% and 19.3%, respectively, for active metabolite AUC, and 30.1% and 38.1% respectively, for active metabolite C_{max} .

It was found that two 5-mg prasugrel hydrochloride tablets were bioequivalent with one 10-mg prasugrel hydrochloride tablet. During tablet manufacturing and storage, prasugrel hydrochloride tablets can convert to prasugrel base. The conversion from salt to base up to 70% has no impact on the extension and rate of the bioavailability of prasugrel at normal gastric pH, and furthermore, as study TACR confirmed, a 5 to 70% conversion of prasugrel hydrochloride tablets to prasugrel base did not affect the AUC or C_{max} of R-138727 in healthy subjects with normal gastric pH. There is a procedure in place with the aim of controlling the conversion and keeping it within this rate. The influence of food was assessed with a 25 mg and 15 mg dose of prasugrel. One of the effect of food on R-138727 AUC was the lower absorption rate, with C_{max} being 48.8% lower in the fed state, t_{max} delayed from 0.5 to 1.5 hours. Although an important pharmacokinetic parameter during maintenance dose is AUC, C_{max} is considered relevant in patients who receive a loading dose in order to achieve a more rapid onset of the pharmacological effect. Although the percutaneous coronary intervention (PCI) is usually performed in the fasted state, it is necessary to point out the importance of the administration of prasugrel loading dose in fasted state in the SPC. The CHMP thus proposed to revise the SPC wording to reflect that the onset of action of the loading dose may be most rapid in the fasted state and this new wording was accepted.

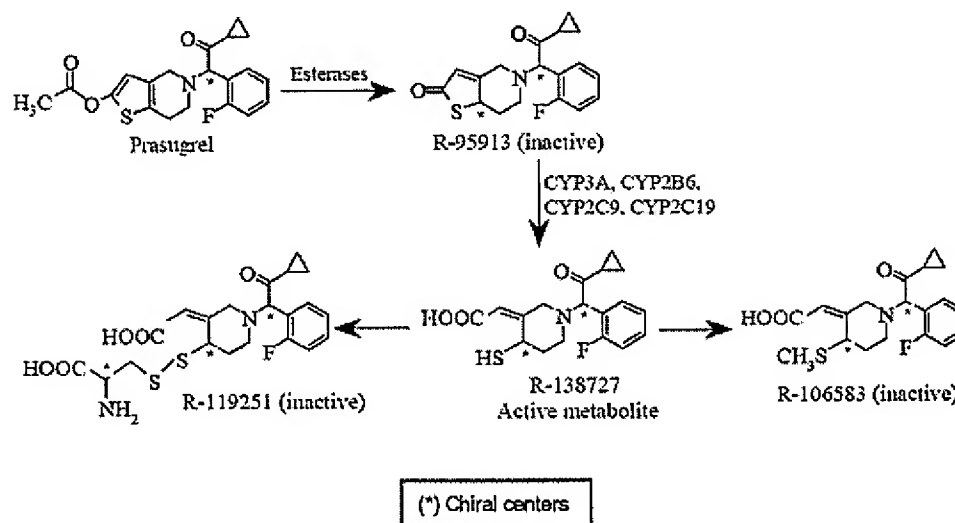
- **Distribution**

Estimates of apparent volume of distribution of R-138727 ranged between 40.3-66.4 l in healthy subjects and subjects with stable atherosclerosis. Prasugrel metabolites demonstrated limited penetration into red blood cells and the plasma-to-whole blood ratio was generally greater than one suggesting that radioactivity in the plasma was greater than that in an equivalent volume of blood cells. Because R-138727 is unstable in plasma, its binding to plasma proteins could not be determined. However, in a 4% human serum albumin solution in phosphate buffer at pH 7.4, R-138727 was 98% bound. For the inactive metabolites, the fraction bound to plasma proteins at various concentrations determined by ultracentrifugation, was 94.6% for R-95913, 95.1% for R-106583, and 76.4% for R-119251. Thus, the active metabolite is highly bound to protein and the measured concentration will depend on the protein content, which may be influenced by factors such as renal function, age and concomitant medication. However, only a minor fraction is unbound and this is not likely to change

significantly. Although the total concentration of the active metabolite might be lower, the effect of the drug may be similar patients with renal failure to that found in healthy persons.

- Elimination

Prasugrel is *in vivo* rapidly hydrolysed by esterases and the product of this hydrolysis, the pharmacologically inactive thiolactone R-95913, is metabolised to the active metabolite R-138727 mainly by cytochrome P450 CYP3A and CYP2B6, and, to a lesser extent, by CYP2C9 and CYP2C19. R-138727 is further metabolised to two inactive compounds by S-methylation or conjugation with cysteine (R-119251 and R-106583). Other prasugrel metabolites are formed by oxidation and/or conjugation and are not pharmacologically active. In case of the active metabolite R-138727, which is eliminated by S-methylation and conjugation with cysteine, it is unclear which enzyme is involved in the elimination of the active metabolite. The CHMP was concerned about the clinical relevance of this issue and requested further clarifications. Based on the *in vitro* study, it would appear that thiopurine S-methyltransferase (TPMT) is not responsible for the S-methylation of R-138727 to R-106583 and that the S-methylation appeared mainly in human liver microsomes. Formation of R-106583 was inhibited by an inhibitor of thiol S-methyltransferase (TMT). The results thus suggest that TMT, and not TPMT, is responsible for R-106583 formation from prasugrel's active metabolite in human liver. However, possible inhibition of TMT by other drugs is unknown. It is considered beneficial that the rapid and efficient generation of the active metabolite of prasugrel results in its rapid appearance in plasma and consequently, in a rapid and extensive inhibition of platelet aggregation. Prasugrel exposure appears to be essentially unaffected by CYP inhibitors, inducers, and competitive inhibition by CYP substrates.



Simplified prasugrel metabolic pathway.

Approximately half of the active metabolite amount appearing in plasma is formed during absorption and/or during first-pass metabolism in liver, which explains the rapid appearance of active metabolite in plasma. Other prasugrel metabolites are formed by oxidation and/or conjugation and are not pharmacologically active.

Approximately 95% of a [^{14}C]prasugrel dose was recovered after oral administration. It was estimated that ca 68% of the prasugrel dose is excreted in urine and 27% in faeces in form of the inactive metabolites over a period of 10 days. Thus, urinary excretion is the major pathway for the elimination of prasugrel metabolites. The elimination half-life of R-138727 is about 7.4 hours. No R-138727 is detected in urine or faeces.

- Dose proportionality and time dependencies

Time dependency has not been specifically addressed. Several clinical studies support the evidence that exposure to prasugrel's active metabolite is dose-proportional. Furthermore, the comparison of $AUC_{(0-4)}$ and C_{max} values for the active metabolite with the dose shows a linear relationship with no discernable deviation from linearity over the entire dose range of 5 - 60 mg.

- Special populations

Impaired renal function

The effect of renal impairment on the disposition of prasugrel metabolites and platelet aggregation was investigated in three clinical studies (TAAO, TABW, and TACJ). Included were subjects with end stage renal disease, subjects with moderate renal impairment

Moderate Renal Impairment and End Stage Renal Disease (ESDR)

The $AUC_{(0-last)}$ and C_{max} values for the active metabolite R-138727 both averaged 38% lower in subjects with ESRD on dialysis across the dose range of 5- 60mg than in subjects with normal renal function. The lower active metabolite exposure in subjects with ESRD is generally consistent with an analysis across all three studies, TAAO, TABW and TACJ, in subjects with ESRD who received a 60-mg LD of prasugrel. Despite the differences in active metabolite exposure, platelet aggregation response to prasugrel is similar in ESRD and healthy subjects. Although subjects older than 65 typically have some degree of renal impairment, no differences in AUC or C_{max} of the active metabolite were observed in a clinical setting. Exposure to the active metabolite was comparable in subjects with moderate renal impairment (estimated creatinine clearance of 30-50 mL/min) and matched healthy controls; although median exposure to prasugrel's active metabolite was higher by approximately 22% in subjects with mild renal impairment than in subjects with normal renal function. The analyses of subjects with renal impairment in clinical pharmacology studies and in the substudy in phase 3 trial do not support the need for a dose adjustment for renal impairment. The CHMP, however, requested more information regarding the observed inconsistency in the pharmacokinetic parameters, especially as patients with end stage renal function seem to have lower levels of the active metabolite compared to healthy subjects. Plausible explanations for the comparable efficacy between ESRD patients and healthy adults were provided and these do not suggest that the dose adjustments are warranted. Nevertheless, the risk of bleeding episodes may be increased in patients with ESRD and the need for caution is reflected in the SPC.

Impaired hepatic function

Two Studies were performed in patients with mild to moderate hepatic function (Child-A and Child-B). Based on these results no dose adjustment in this population appears necessary, however, caution should be exercised in patients with moderate hepatic impairment. Clinical trials performed with prasugrel have not included patients with severe hepatic impairment (Child-C). As this population has a higher risk of bleeding, a contraindication in the SPC for patients with severe hepatic impairment (Child Pugh Class C) was included.

Gender

The pharmacokinetic meta-analysis of 16 clinical pharmacology studies detected no effect of gender on the exposure to prasugrel's active metabolite.

Race

The effect of ethnic origin was assessed in the pharmacokinetic meta-analysis of 16 clinical pharmacology studies. Most of the 437 subjects evaluated after a prasugrel LD and 284 subjects evaluated during prasugrel MD were Caucasian, although about 22% were Asian. Most Asian subjects in the meta-analysis originated from the three clinical pharmacology studies specifically designed to assess the influence of Asian ethnicity on prasugrel pharmacokinetics and pharmacodynamics. In each of these studies, Caucasian subjects served as the reference population.

Active metabolite exposure was similar in Chinese, Japanese, and Korean subjects after a 60-mg LD and during 10-mg and 5-mg MDs. However, the analysis showed that $AUC_{(0-last)}$ in Asians was 40% higher during MD and compared to Caucasians, the higher exposures in Asians produced higher inhibition of platelet aggregation (IPA) at most time points. Asians and Caucasians in the LD portion

of the pharmacokinetic meta-analysis had mean body weights of 65 kg and 77 kg, respectively, so the meta-analysis compared weight normalised $AUC_{(0-t_{last})}$ and C_{max} values between Asians and Caucasians to assess the contribution of weight to exposure. After adjusting for body weight, the $AUC_{(0-t_{last})}$ of the active metabolite was still approximately 19% higher in Chinese, Japanese, and Korean subjects compared to that of Caucasians, predominantly related to higher exposure in Asian subjects <60 kg. No dose adjustment is recommended based on ethnicity alone, but therapeutic experience with prasugrel is limited in Asian patients and therefore, prasugrel should be used with caution.

Weight

Analyses of several clinical studies in healthy subjects, subjects with stable atherosclerosis and subjects with acute coronary syndrome undergoing PCI consistently show that the AUC of the prasugrel active metabolite increases with a decreasing body weight. The relationship between the body weight and the active metabolite AUC was assessed using a conventional statistical approach that relied on univariate and multivariate analyses to quantify the magnitude of the body weight effect on active metabolite exposure. In healthy subjects, weight was one of the two covariates declared clinically significant in a multivariate analysis of these data, the other being Asian ethnicity. The univariate analysis supports consideration of dose adjustment at any weight threshold from ≥ 55 kg through 80 kg, while the multivariate analysis supports dose adjustment consideration at any weight threshold from ≥ 50 kg through 80 kg. In subjects with ACS undergoing PCI in Study TAAL, weight was one of the three covariates declared significant during a multivariate analysis of these data, the other two being age and gender. The univariate analysis of the body weight effect supports consideration of dose adjustment for subjects <70 kg, but not for subjects ≥ 75 kg. The multivariate analysis of the body weight effect supports dose adjustment consideration for subjects <55 kg, but not for subjects ≥ 59 kg. The similarity in conclusions between the univariate and multivariate analyses clearly show that body weight is an important covariate. Further analyses of the risk for TIMI bleeding by different weight indicate that the odds ratio for bleeding with 10 mg prasugrel increases rapidly in the vicinity of 60 kg (and 75 years of age); supporting these values as cut-off choices for dose adjustment. A PK/PD model to assess the effect of the reduced dose (5 mg) in subjects < 60 kg or ≥ 75 years of age was developed and although the results are reassuring, clinical confirmation is needed (please see section Clinical Efficacy). Thus, the CHMP accepted the follow up measure to conduct a clinical study in subjects with stable CAD to compare the PK, PD, safety, and tolerability of prasugrel in subjects <60 kg to that of subjects ≥ 60 kg. Subjects will be treated with a maintenance dose of either prasugrel 5-mg, prasugrel 10-mg, or clopidogrel 75-mg. This study will exclude subjects ≥ 75 years. In addition, the SPC advises that the 10 mg maintenance dose is not recommended in subject weighing <60 kg.

Elderly

Age was one of 3 covariates declared statistically significant during a multivariate analysis of TAAL data as described above. When exposure was normalized by body weight, the 90%CI for the effect of age was below 1.25 for all age thresholds from 50 to 80 years old. Despite the lack of relationship between age and AUC in the multivariate analyses above, safety analyses of study TAAL revealed a strong relationship between bleeding risk and age, with a higher rate of bleeding in subjects ≥ 75 years old compared to those <75 years old. This prompted more extensive assessments of active metabolite exposure in the elderly.

The analysis focused on exploring the differences in exposure in patients approximately at and below the median age in the TAAL study compared to exposure in patients whose age was above specific thresholds up to 85 years. Based on this, a consideration of dose adjustment should be made at 70 years and more, with specific dose recommendations and the age thresholds associated with those recommendations depending on safety. Furthermore, an assumption was made about the anticipated clinical use of prasugrel where patients <60 kg would receive a 5-mg MD rather than a 10-mg MD, and patients ≥ 60 kg would then be considered for dose adjustment based on their actual age. In this approach a univariate analysis of age effect in subjects ≥ 60 kg is clinically more relevant and when this subgroup of patients ≥ 60 kg is assessed, the active metabolite AUC for patients ≥ 74 years is significantly larger than that for patients <74 years. Consistent with this difference, more than 60% of

patients ≥ 60 kg and ≥ 75 years old had concentrations above the median exposure in the TAAL study. This supports consideration of dose adjustment at any age threshold of 75 years or older, although specific dose recommendations and the age thresholds associated with those recommendations depend on safety.

In summary, age is a significant risk factor for bleeding. A cut-off level of 75 years based on a pharmacokinetic univariate analyses in subjects ≥ 60 kg is suggested.. This issue was addressed during the Scientific Advisory Group (SAG) meeting and in the oral explanation held at the CHMP meeting (please see section on Clinical Efficacy). Based on the CHMP discussions following the SAG meeting, Company written responses and oral explanation, the CHMP requested a strict SPC wording, which advises that the use of prasugrel in patients ≥ 75 years of age is generally not recommended. If use is deemed necessary based on careful individual benefit/risk evaluation by the prescribing physician, then following a 60 mg loading dose a reduced maintenance dose of 5 mg should be prescribed. An educational programme with regard to this topic is part of the conditions for the safe and effective use of the product (see sections 2.4 and 2.5). The results of the analysis conducted *via* a PK/PD model to evaluate the dose 5 mg in patients < 60 kg or ≥ 75 years of age need clinical confirmation and the Company is conducting such studies as part of the follow-up measures..

- Pharmacokinetic interaction studies

In vitro, prasugrel metabolites R-138727 and R-106583 did not inhibit the activities of cytochrome P450 CYP2D6, CYP2C9, CYP2C19, CYP1A2 and CYP3A4 hepatic isoforms up to 200 μ M. The other major metabolite, the hydrolysis product R-95913, did not inhibit CYP1A2, but did inhibit CYP2D6, CYP2C9, CYP2C19 and CYP3A4. The projected maximum inhibition ranged from 2% for CYP2C9 to 21% for CYP2C19. None of these effects were deemed as a cause of a significant effect in the clearance of drugs metabolised by these enzymes. The effect of prasugrel on CYP1A2 and CYP3A4 was also assessed in primary cultures of human hepatocytes from four donors at various concentrations. No effect was observed on CYP1A2, but R-95913 showed a slight to moderate induction of CYP3A4 at a clinically relevant concentration. In order to further assess the clinical consequences of the signals detected from *in vitro* studies, a number of *in vivo* studies, including the assessment of pharmacokinetic interactions with aspirin, ranitidine, ketoconazole (CYP3A4/5 inhibitor), rifampicin (inducer of several CYP enzymes), atorvastatin, bupropion (a CYP2B6 substrate), warfarin, and heparin was conducted. An interaction study with digoxin was also conducted; aiming at the assessment of the effect of prasugrel on P-glycoprotein. Only a slight inhibitory effect of prasugrel on CYP2B6 (decreased hydroxibupropion exposure by around 20-30%) was observed. This effect is likely to be of clinical concern only if prasugrel is co-administered with drugs for which CYP2B6 is the only metabolic pathway and have a narrow therapeutic window. This concern of the CHMP is adequately expressed into the SPC of this medicinal product. Furthermore, inhibition or induction of CYP3A4 enzyme did not indicate any significant effect on prasugrel. Co-administration of prasugrel with digoxin at steady state did not significantly affect digoxin renal clearance and overall pharmacokinetics. Furthermore, prasugrel showed a lack of influence on the pharmacokinetics of S-warfarin, but caution should be exercised when prasugrel and warfarin are given together due to the potential increased risk of bleeding. Similarly, additional consideration is necessary during the co-administration of prasugrel with heparin as stated in SPC ("an increased risk of bleeding is possible when Eflent is co-administered with heparin"). Daily co-administration of products elevating the gastric pH value, e.g. ranitidine or lansoprazole, did not change the metabolite's AUC and T_{max} , but decreased the C_{max} by 14% and 29%, respectively. Although in the maintenance therapy the C_{max} changes could be considered of less clinical relevance, in the loading dose when the intention is to achieve maximum inhibition of the platelet aggregation as quickly as possible, the C_{max} is considered a clinically relevant parameter. Therefore, a recommendation in the SPC that administration of the loading dose without concomitant administration with proton pump inhibitors may provide most rapid onset of action was included. In summary, the potential for pharmacokinetic interactions with prasugrel was adequately studied both *in vitro* and *in vivo*.

Pharmacodynamics

Platelets play a central role in the pathogenesis of atherothrombosis and in the formation of thrombi following coronary angioplasty, with and without stent implantation. Platelets initially adhere at sites of vascular injury, atherosclerotic plaque rupture, balloon angioplasty, and stenting. Platelet activation following these interactions results in the release of adenosine diphosphate (ADP), thromboxane A₂, and other mediators. Released ADP promotes platelet activation *via* the G-protein linked P2Y₁ and P2Y₁₂ purinergic receptors leading to further platelet activation, aggregation, and other platelet functions, such as platelet shape change, secretion, and the development of pro-coagulant and pro-inflammatory activities. Activated platelets are recruited to sites of coronary plaque rupture and intra-arterial stenting, thereby forming aggregates that may lead to platelet-rich thrombi, vascular occlusion, tissue ischemia, and myocardial necrosis in what is collectively known as acute coronary syndromes.

Prasugrel is a thienopyridine ADP receptor antagonist that irreversibly inhibits platelet activation and aggregation mediated by the P2Y₁₂ receptor. Prasugrel has a distinct chemical structure that permits efficient conversion to its active metabolite through rapid hydrolysis by carboxylesterases and then by multiple CYP450 enzymes.

- Mechanism of action

Prasugrel's pharmacological action is analogous to that described for other thienopyridines and results from covalent and irreversible binding of the active metabolite R-138727 to the P2Y₁₂ platelet ADP receptor. Once bound, a platelet is rendered ineffective for its remaining lifespan. After prasugrel administration is stopped, return to baseline platelet aggregation occurs only as new platelets are formed. The return to the baseline typically occurs at about 7 - 10 days after treatment is interrupted.

- Primary and Secondary pharmacology

Four initial studies assessing the safety, pharmacokinetic and pharmacodynamics of prasugrel in small numbers of healthy subjects allowed for the initial assessment of prasugrel activity, but did not analyze for prasugrel's active metabolite. Subsequently, four studies aimed at characterisation of the prasugrel hydrochloride salt and four initial clinical studies were conducted with prasugrel base to characterise pharmacokinetic, pharmacodynamics and tolerability in healthy subjects. Pharmacodynamics effects of thienopyridines on platelet function may be assessed by inducing platelet aggregation with various concentrations of ADP. Response to 20 µM ADP has been used as the primary pharmacodynamic parameter considering that it is a specific indicator of P2Y₁₂ function. Four clinical studies were conducted to evaluate the prasugrel-mediated inhibition of platelet aggregation and to characterise prasugrel's safety and tolerability, its pharmacokinetic and pharmacodynamic profile, effects on platelet function and bleeding time. In all tests, effective inhibition of platelet aggregation was observed with the onset of effect occurring within 1 hour of dosing. The effect continued through 48 hours post dosing. Platelet aggregation returned to normal levels at day 7. Reported adverse events included gastrointestinal disturbances, autonomic disturbances and general disorders, but none were serious.

A meta-analysis of pharmacodynamic data across the studies in healthy subjects and in subjects with stable atherosclerosis was conducted (see figure below) and the results indicate that within 30 minutes, the average inhibition of platelet aggregation (IPA) exceeds 50%. This time point is a key value, because it is the first assessed time point at which the IPA shows a statistically significant difference from baseline. Furthermore, within 1 hour, 97% of the subjects achieved an IPA above 20%, with the average IPA exceeding 70%. Over 89% of all subjects achieved at least 50% IPA by 1 hour, and over 90% of the maximum mean IPA is achieved by that time. One hour is a relevant time point because the average IPA across all subjects after a prasugrel 60-mg LD is nearly as high as the peak IPA eventually reached. By 4 hours, the average IPA is about 80%. More than 99% of the prasugrel subjects in the meta-analysis had an IPA above 20%, and about 90% of subjects achieved 90% of their individual maximum IPA by then. At each of these time points, a 300-mg clopidogrel LD showed a lower peak of IPA, fewer subjects achieved ≥20% IPA. The results in response to 5 µM ADP are similar. Following the administration of a single dose of prasugrel to healthy subjects not taking acetylsalicylic acid, platelet aggregation returned to normal levels by day 6 after a single administration of 30- or 75-mg dose of prasugrel base. After multiple doses of prasugrel to healthy

subjects taking acetylsalicylic acid, platelet aggregation returned to baseline levels in 5 days following discontinuation of MD at steady-state.

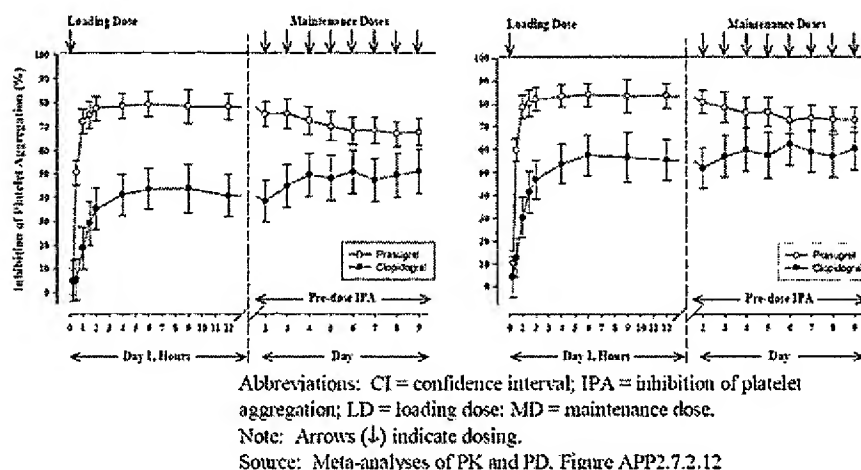


Figure 2.7.2.10. Least squares mean ($\pm 95\%$ CI) IPA to 20 μM (left panel) and 5 μM (right panel) after prasugrel 60-mg LD/10-mg MD and clopidogrel 300-mg LD/75-mg MD – PD Meta-analysis.

In summary, the results of the evaluation of the pharmacodynamic effects of prasugrel as an inhibitor of platelet aggregation were expressed as maximum platelet aggregation (MPA), which decreases with increasing pharmacodynamic response, and IPA, which is derived from the MPA determination and increases with increasing pharmacodynamic response. Clinical studies comparing the pharmacodynamic response of prasugrel with that of clopidogrel at the loading or loading/maintenance doses showed that the maximum mean IPA was achieved faster and was greater with prasugrel. Greater pharmacodynamic response for prasugrel is believed to be the result of the more rapid and more extensive formation of its active metabolite and has a less response variability compared to clopidogrel.

No relevant pharmacodynamic interactions were noticed when prasugrel is coadministered with unfractionated ranitidine, ketoconazole, atorvastatin, unfractionated heparin, digoxin and warfarin. There is an additive pharmacodynamic interaction between aspirin and prasugrel, in terms of suppression of platelet aggregation induced by collagen. The pivotal evidence of prasugrel in the claimed indication has been obtained as an add on therapy to low dose aspirin and thus, the potential safety risk of this interaction has been evaluated. In addition, the metabolic pathways for aspirin are separate from those for prasugrel and therefore no metabolic interaction would be expected. Co-administration of ketoconazole, a potent inhibitor of CYP3A4 and CYP3A5, with prasugrel did not significantly affect the exposure of the active metabolite of prasugrel, or the drug's effect on platelet inhibition. The possibility that the pharmacokinetics of prasugrel could be affected by inhibiting two or more pathways involved in prasugrel's metabolism was considered; however, only ticlopidine was listed as an acceptable CYP2B6 inhibitor. In addition, clopidogrel is the other drug that was clinically shown to be potent, mechanism-based inhibitor of CYP2B6. Because co-administering either of these drugs with prasugrel would not be considered in clinical practice, clinical evaluation of concomitant administration of prasugrel and of CYP2B6 (ticlopidine or clopidogrel) was not conducted. A clinical study aiming to assess the effect of 80 mg of prasugrel (single dose) on cardiac repolarisation was conducted including placebo and moxifloxacin as positive controls. The study design was in agreement with the ICH-E14 guideline. No effect of prasugrel on QT was observed above the threshold of regulatory concern (10 msec) at any time point. The positive control (moxifloxacin) showed a maximum QT prolongation effect within the expected range.

Clinical efficacy

The following studies have been conducted in order to support the use of prasugrel for the reduction of atherothrombotic events (CV death, nonfatal MI, or nonfatal stroke) in subjects with ACS:

Study Alias	Study Type	Subjects (N)	Primary Objective	Overall Conclusions
H7T-MC-TAAH	Phase 2 Dose Ranging Safety (multiple LD/MD regimens) Prasugrel (40-mg LD, 7.5-mg MD) Prasugrel (60-mg LD, 10-mg MD) Prasugrel (60-mg LD, 15-mg MD) Clopidogrel (300-mg LD, 75-mg MD): All treatments were co-administered with aspirin. 30-day duration	Elective and urgent PCI (905)	1) Evaluate the safety of increasing doses of prasugrel by observing the rate of Non-CABG-associated significant bleeding (that is, Major plus Minor bleeding at 30 to 35 days after PCI). 2) Compare the safety of prasugrel to a standard regimen of clopidogrel (a 300-mg LD during PCI and 29 to 34 days of a 75-mg once daily MD) by observing the rate of Non-CABG-associated significant bleeding 30 to 35 days after PCI	60-/10-mg LD/MD prasugrel showed comparable TIMI Major + Minor bleeding to 300-/75-mg LD/MD clopidogrel, trend towards decreased 30-day MACE
H7T-MC-TABL	Phase 2 Comparative PD (60-/10-mg LD/MD prasugrel vs 600-/150-mg LD/MD clopidogrel regimens). 30-day duration	Elective PCI (201)	1) To compare the inhibition of platelet aggregation (IPA) with 20 μ M ADP measured at 6 hours (\pm 30 minutes) after prasugrel 60-mg LD versus clopidogrel 600-mg LD in subjects who did not receive a GP IIb/IIIa antagonist. 2) To compare the IPA with 20 μ M ADP measured after 14 \pm 2 days of prasugrel 10-mg daily MD versus the IPA after 14 \pm 2 days of clopidogrel 150 mg daily MD in the "on-treatment population" who received PCI regardless of GP IIb/IIIa antagonist use (this included subjects receiving prasugrel and clopidogrel, in either order, during crossover)	60-/10-mg LD/MD prasugrel showed more rapid onset of higher IPA versus 600-/150-mg LD/MD clopidogrel
H7T-MC-TAAL	Phase 3 Pivotal Study (60-/10-mg LD/MD prasugrel vs 300-/75-mg LD/MD clopidogrel regimens) with aspirin. Maximum duration 15 months	PCI in ACS (13608)	To demonstrate superiority of prasugrel co-administered with aspirin relative to clopidogrel co-administered with aspirin, as measured by a reduction in the composite endpoint of CV death, onfatal MI, or nonfatal stroke at a median follow up of at least 12 months.	Superior efficacy for 60-/10-mg LD/MD prasugrel vs 300-/75-mg LD/MD clopidogrel regimens with higher risk of bleeding

Abbreviations: ACS = acute coronary syndromes; CAD = coronary artery disease; IPA = inhibition of platelet aggregation; LD = loading dose; MACE = major adverse cardiovascular events; MD = maintenance dose; N = number randomly assigned to prasugrel and/or clopidogrel; PCI = percutaneous coronary intervention; PD = pharmacodynamic; PK = pharmacokinetic; PK/PD = pharmacokinetic/pharmacodynamic; TIMI = Thrombolysis In Myocardial Infarction.

- Dose response studies

Phase 3 dose selection was based primarily on 2 randomized clinical studies in subjects with stable atherosclerosis using the approved clopidogrel 300-/75-mg LD/MD regimen as the active comparator. The first study (TAAD) was a 28-day, phase 1b, dose-ranging pharmacokinetic/pharmacodynamic study in aspirin-treated subjects (N=101) comparing platelet inhibition using standard aggregometry. The second study (TAAH) was a 30-day, phase 2, dose-ranging safety study in aspirin-treated subjects (N=905) undergoing elective or urgent PCI (see below). Study TABL was conducted in parallel with the pivotal study TAAL to investigate the safety and pharmacodynamics of prasugrel against higher dose regimens of clopidogrel.

Study TAAD

This was a 28-day, phase 1b, dose-ranging pharmacokinetic/pharmacodynamic study in stable atherosclerosis patients (N=101) treated with aspirin (375 mg), in which the platelet inhibition was compared using the standard aggregometry. It is worth noting that the participants in this study are not entirely representative of those claimed in the indication of the current submission. In this study four different regimens (40 mg/5 mg, 40 mg/7.5 mg, 60 mg/10 mg and 60 mg/15 mg) were compared with the approved clopidogrel LD/MD regimen (300mg/75 mg). Overall, both the 40-mg and 60-mg prasugrel LDs resulted in more rapid onset with significantly greater IPA to 20 μ M ADP from 2 to 6 hours after administration than the 300-mg LD of clopidogrel. Both the 10- and 15-mg prasugrel MDs achieved consistent and significantly greater IPA than the 75-mg clopidogrel MD; however, the 15-mg MD of prasugrel was associated with higher bleeding adverse events. In contrast, the prasugrel 5- and 7.5-mg MD groups were not consistently and statistically different in IPA from the clopidogrel 75-mg MD group.

Study TAAH

This was a double-blind, randomized, multicentre, dose-ranging trial of prasugrel compared with clopidogrel in subjects undergoing PCI. The primary endpoints evaluated the safety of increasing doses of prasugrel (a loading dose during PCI and once-daily maintenance dosing for 29 to 34 days) and compared prasugrel's safety with a standard regimen of clopidogrel (300-mg LD during PCI and 75-mg once-daily maintenance dose for 29 to 34 days) by observing the rate of non-CABG-associated significant bleeding (i.e. major and minor bleeding at 30 to 35 days after PCI).

The overall observed rate of all bleeding events was higher for subjects in the combined prasugrel group (29/650 subjects, 4.5%) compared with subjects in the clopidogrel group (9/254 subjects, 3.5%), but this difference was not statistically significant. With regard to the bleeding events, neither the differences among prasugrel dose groups ($p=0.933$), nor the differences between prasugrel and clopidogrel ($p=0.590$) were statistically significant. Thus, it was concluded that there was no statistically significant difference in the safety of increasing doses of prasugrel and no statistically significant difference between the safety of prasugrel and the standard clopidogrel regimen. The overall rate of non-CABG-associated significant bleeding was lower than anticipated and this resulted in reduced statistical power to assess the safety of prasugrel. A reduction of dose in the very elderly was recommended based on the pivotal study and is described later.

- Main studies

The pivotal phase 3 Study (H7T-MC-TAAL, further referred to as TAAL) was a global, multicentre, parallel-group, randomized, double-blind, double-dummy, active-controlled study. The primary objective of Study TAAL was to test the hypothesis that prasugrel is superior to clopidogrel in the treatment of subjects with ACS undergoing PCI, as measured by a reduction in the primary composite efficacy endpoint of CV death, nonfatal MI, or nonfatal stroke. This study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki and that are consistent with good clinical practices (GCP) and the applicable laws and regulations of where the study was conducted. Study TAAL was evaluated based on several relevant guidelines:

- The CONSORT statement (The Lancet 2001;357:1191-94),
- Points to consider on the clinical investigation of new medicinal products for the treatment of unstable angina pectoris or non-Q-wave myocardial infarction, CPMP/EWP/570/98,
- Points to consider on application on one pivotal study (CPMP/EWP/2330/99),
- Points to consider on the clinical development of fibrinolytic medicinal products in the treatment of patients with ST segment elevation acute myocardial infarction (STEMI), CPMP/EWP/967/01.

In addition, scientific advice given by the CHMP in 2004 was considered when planning this pivotal study.

METHODS

Study Participants

Participants were to be of a legal age (and at least 18 years of age) and competent mental condition to provide written informed consent. For women of child-bearing potential, only those tested negative for pregnancy between ACS presentation and enrolment and agreed to use a reliable method of birth control during the study were included. Subjects with ACS (subjects with unstable angina and non-ST-segment elevation myocardial infarction [UA/NSTEMI] with TIMI risk score ≥ 3 or ST-segment elevation myocardial infarction [STEMI]) who are to undergo PCI were eligible to enter the study. The main inclusion criteria were:

- Moderate- to high-risk UA was defined as a history of chest discomfort or ischemic symptoms of ≥ 10 minutes duration at rest ≤ 72 hours prior to randomization, with persistent or transient ST-segment deviation ≥ 1 mm in one or more ECG leads without elevation of CK-MB or troponin T or I but with a TIMI risk score ≥ 3 .
- Moderate- to high-risk NSTEMI was defined as a history of chest discomfort or ischemic symptoms of ≥ 10 min duration at rest ≤ 72 hours prior to randomization with no evidence of persistent ST-segment elevation. Subjects must also have CK-MB or troponin T or I greater than the upper limit of normal (ULN) and a TIMI risk score ≥ 3 .
- ST-segment elevation myocardial infarction (STEMI) is defined as a history of chest discomfort or ischemic symptoms of >20 minutes duration at rest ≤ 14 days prior to randomization with one of the following present on at least one ECG prior to randomization: a) ST-segment elevation ≥ 1 mm in two or more contiguous ECG leads. b) New or presumably new left bundle branch block (LBBB). c) ST-segment depression ≥ 1 mm in two anterior precordial leads (V1 through V4) with clinical history and evidence suggestive of true posterior infarction.

Subjects with STEMI could be enrolled within 12 hours of symptom onset if primary PCI was planned or within 14 days if delayed PCI was planned following initial pharmacotherapy for STEMI.

Exclusion criteria were extensive. In general, these excluded subjects at increased risk of bleeding (for example, anaemia, thrombocytopenia, intracranial pathology, severe hepatic dysfunction, oral anticoagulants, chronic non-steroidal anti-inflammatory drug (NSAID) use, or use of any thienopyridine within 5 days of the main treatment), patients with refractory ventricular arrhythmia, class IV congestive heart. The inclusion/exclusion criteria are considered acceptable. Diagnosis and short-term risk stratification is based on the combination of ischaemic symptoms, ECG changes, biomarkers in some cases and risk score results. The recommendations of the European Society of Cardiology guidelines (2007) were followed.

Treatments

This study involved a comparison of prasugrel (60-mg LD, 10-mg MD) and clopidogrel (300-mg LD, 75-mg MD). Both treatments were administered orally as a one-time LD followed by a once daily MD. The loading and maintenance doses of prasugrel for this Phase 3 PCI study were selected on the basis of non-clinical thrombosis models, non-clinical toxicology studies, dose-escalation studies in healthy subjects, a dose-ranging study versus clopidogrel in subjects with stable coronary artery disease (Study TAAAD), and a dose-ranging study versus clopidogrel in subjects undergoing elective or urgent PCI (Study TAAH). Owing to the link observed between thrombosis complications following PCI and poor antiplatelet response to clopidogrel, recommendations for the use of doses higher than the standard in PCI have been reported. There is evidence of some increase in the speed of onset and the level of platelet inhibition with both 600 mg and 900 mg of clopidogrel LDs. Still, these assumptions are based on limited data and require further sound confirmation. Thus, the use of the standard 300 mg LD (administration as soon as possible) and 75 mg/day MD of clopidogrel is acceptable.

Objectives

Primary objective: To test the hypothesis that prasugrel (co-administered with aspirin) was superior to clopidogrel (co-administered with aspirin) in the treatment of subjects with acute coronary syndromes who were to undergo percutaneous coronary intervention, as measured by a reduction in the composite endpoint of cardiovascular (CV) death, nonfatal myocardial infarction (MI), or nonfatal stroke at a median follow-up of at least 12 months.

The secondary efficacy objectives were to compare both treatments with respect to the:

- risk of cardiovascular (CV) death, nonfatal myocardial infarction (MI), or nonfatal stroke through 90 days.
- risk of CV death, nonfatal MI, or nonfatal stroke through 30 days.
- risk of CV death, nonfatal MI, or urgent target vessel revascularization through 90 days.
- risk of CV death, nonfatal MI, or urgent target vessel revascularization through 30 days.
- risk of all-cause death, nonfatal MI, or nonfatal stroke at study end.
- risk of CV death, nonfatal MI, nonfatal stroke, or rehospitalisation for cardiac ischemic events at study end.
- risk of definite or probable stent thrombosis per Academic Research Consortium (ARC) definition at study end.

The safety objectives of this study were to compare prasugrel with clopidogrel with respect to the:

- risk of non-coronary artery bypass graft (Non-CABG) Thrombolysis in Myocardial Infarction Study Group (TIMI) Major bleeding in subjects receiving prasugrel or clopidogrel.
- risk of Life-Threatening bleeding (a subset of Non-CABG-related TIMI Major bleeding) in subjects receiving prasugrel or clopidogrel.
- risk of Non-CABG-related TIMI Minor bleeding in subjects receiving prasugrel or clopidogrel.
- The overall safety and tolerability of prasugrel administration based on clinical findings, laboratory values, and the occurrence of treatment-emergent adverse events.

Outcomes/endpoints

The primary efficacy measure was time to first event of a composite of cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke at study end. Cardiovascular Death (CV Death) was defined as death due to documented cardiovascular cause. Additionally, death not clearly attributable to non-cardiovascular causes was considered CV death. The definition of myocardial infarction (MI) was adapted from the standard American College of Cardiology (ACC) definition and was dependent on the clinical timing of the event in relation to presenting syndrome and cardiovascular procedures. A peri-procedural event must be distinct from the index event. If an ischemic biomarker was elevated at the onset of the suspected event, there must be demonstration of a falling biomarker level prior to the onset of the suspected event, and the subsequent peak must be greater than 1.5 times the value prior to the onset of the event. The biomarker levels required for the diagnosis of MI were dependent on relationship to cardiac procedures:

- If the suspected event was within 48 hours of a PCI, the CK-MB value (on at least two samples) must be $>3\times$ ULN or $>5\times$ ULN on the last available sample provided it was obtained ≥ 12 hours after the PCI; no symptoms were required.
- If the suspected event was within 48 hours of a CABG, the CK-MB value (on a single measure) must be $>10\times$ ULN; no symptoms were required.
- If the suspected event was not within 48 hours of a PCI or CABG, the diagnostic criteria were met if the subject had CK-MB or cardiac troponin $>$ ULN and the presence of either chest pain ≥ 20 minutes in duration or ST-segment deviation ≥ 1 mm on the ECG.

In any clinical circumstance, the appearance of new Q-waves on the ECG distinct from a prior event or pathologic evidence (such as autopsy) showing a new MI felt to be distinct from a prior event were considered appropriate evidence for MI, as would ST-segment elevation lasting for at least 20 minutes and accompanied by ischemic chest pain or haemodynamic decompensation. Nonfatal stroke was defined as the rapid onset of new, persistent neurologic deficit lasting more than 24 hours. In the case of clinical diagnosis of nonfatal stroke, computed tomography (CT) or magnetic resonance imaging (MRI) scan imaging was strongly recommended. CT or MRI scans were considered by the Clinical Events Committee (CEC) to support the clinical impression. Supplemental information from head CT or MRI scans determined if there was a demonstrable lesion compatible with an acute nonfatal stroke. Furthermore, nonfatal stroke was classified as either ischemic or hemorrhagic based on imaging data, if available, or uncertain cause if imaging data were not available.

Secondary efficacy endpoints included:

- CV death, nonfatal MI, or nonfatal stroke through 30 days and 90 days post randomization.
- CV death, nonfatal MI, or UTVR through 30 days and 90 days post randomization. UTVR required both of the following: PCI or CABG, for recurrent ischemia that, in the investigator's opinion, could not be delayed for more than 24 hours and was defined by the investigator as a

- non-elective procedure (urgent), and revascularization, either with CABG or PCI, had to include one or more vessel(s) dilated at the initial (qualifying) procedure.
- All cause death, nonfatal MI, or nonfatal stroke.
- CV death, nonfatal MI, nonfatal stroke, or re-hospitalization for cardiac ischaemic events.
- Definite or probable (Academic Research Consortium (ARC) definition) stent thrombosis.

Safety endpoints were set as follows: Non-CABG-related TIMI Major bleeding, Non-CABG-related TIMI Life-Threatening bleeding, and Non-CABG-related TIMI Minor bleeding.

Non-CABG-related TIMI Major bleeding was any intracranial haemorrhage (ICH) OR any clinically overt bleeding including bleeding evident on imaging studies) associated with a fall in haemoglobin (Hgb) of ≥ 5 gm/dL from baseline. Non-CABG-related TIMI Life-Threatening bleeding was any Non-CABG-related TIMI Major bleeding that was fatal, led to hypotension that required treatment with intravenous inotropic agents, or required surgical intervention for ongoing bleeding, or necessitated the transfusion of 4 or more units of blood over a 48-hour period, or any symptomatic ICH. Non-CABG-related TIMI Minor bleeding was any clinically overt bleeding associated with a fall in Hgb of >3 gm/dL but <5 gm/dL from baseline.

Sample size

Study TAAL was intended to continue until an estimated 875 subjects with UA/NSTEMI reached one of the events described in the triple composite endpoint (CV death, nonfatal MI, or nonfatal stroke) and a median duration of therapy of at least 12 months and a minimum follow-up of 6 months was achieved. A power calculation to assess superiority was performed, assuming a hazard ratio of 0.80 and based on a two-sided log-rank test using a two-sided significance level of 0.05. In view of an anticipated lack of proportionality of hazard, the Gehan-Wilcoxon test was used in the primary analysis. The statistical power of the Gehan-Wilcoxon test depends on the direction and size of the deviation from proportional hazards. It is expected, however, that the power of the Gehan-Wilcoxon test is approximately 90% if the non-proportionality is not severe. A total of around 13,000 subjects with ACS were to be enrolled, so that 9500 subjects with UA/NSTEMI would be enrolled. The number of subjects with STEMI was to be capped at 3500 subjects, which was deemed to be adequate to assess the consistency of treatment effect and safety within the STEMI population.

Randomisation

Subjects were randomized via an interactive voice response system (IVRS) to either prasugrel or clopidogrel in a 1:1 ratio. Randomization was carried out at the site level and stratified by clinical presentation. Subjects were randomized only after diagnostic angiography confirmed anatomy suitable for PCI, except for patients presenting with STEMI with onset of symptoms < 12 hours. Overall, the randomization procedure was assessed as successfully implemented. For a few patients (1.7%) the randomization was based on incorrect strata and the site monitoring identified a small number of patients ($\leq 1.3\%$), who were given the wrong kit/drug. The impact of these protocol violations on the study results is believed to be negligible.

Blinding (masking)

Participants, the sponsors, and all study site personnel were blinded to study drug. Selected clinical study personnel not associated with the study or its operations were granted access to randomization table and treatment assignments. These personnel were limited to those who prepared unblinded summaries and analyses for the periodic safety reviews by the Data Safety Monitoring Board (DSMB) and/or regulatory agencies.

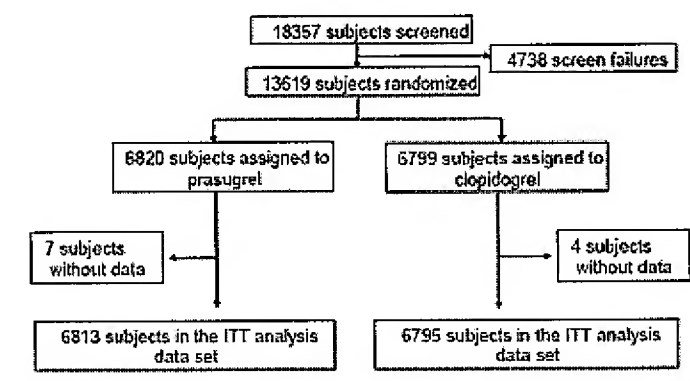
Statistical methods

All efficacy analyses were performed using an intent-to-treat dataset, consisting of all randomized subjects. The safety analyses were carried out using the treated data set that includes all subjects who received at least one dose of study drug, either a loading dose or maintenance dose. Primary efficacy analyses were conducted on endpoints adjudicated by an independent CEC, including CV death, myocardial infarction, stroke, urgent target vessel revascularization, and stent thrombosis. The comparison of the primary endpoint was evaluated using the Gehan-Wilcoxon test from a time-to-first event analysis at a two-sided alpha level of 0.05. Subjects experiencing multiple occurrences of an endpoint were censored at the time of first occurrence. All other key efficacy and safety analyses were

conducted using the log-rank test from a time-to-first event analysis. All efficacy and safety analyses were carried out first for subjects with UA/NSTEMI, followed by analysis of the same endpoint in the All ACS population (UA/NSTEMI plus STEMI subjects) if superiority of prasugrel was demonstrated in the UA/NSTEMI population. The primary outcome was also analyzed in the STEMI population. Analyses including all subjects with ACS included clinical presentation UA/NSTEMI vs STEMI as a stratification factor in the time-to-first event analyses. For the secondary analyses no formal adjustment of the grade of statistical significance is considered necessary, due to the presence of a predefined hierarchical strategy. The statistical analysis plan was well prepared and follows the recommendations in the CHMP Guideline CPMP/EWP/2330/99 (Points to Consider on multiplicity issues and on application with one pivotal study).

Results

Participant flow in study TAAL



There were 7 subjects randomly assigned to prasugrel, and 4 subjects randomly assigned to clopidogrel without data available for inclusion in the final analysis dataset due to an incomplete informed consent document. The most frequent reason for screen failure was that subjects did not meet the inclusion criteria. The majority (98.9%) of subjects completed the study and the number of patients withdrawing consent or considered lost to follow-up was similar between treatment groups.

Recruitment

The enrolment period for Study TAAL was 5 November 2004 to 14 January 2007. The last subject visit occurred on 22 July 2007. The geographic variation, which is likely to depict the future use of the drug, is shown below.

Region of Enrollment n (%) ^a	Prasugrel, n=6813	Clopidogrel, n=6795
North America	2164 (31.8)	2146 (31.6)
United States	2039 (29.9)	2020 (29.7)
Europe	3436 (50.4)	3439 (50.6)
Eastern Europe	1657 (24.3)	1665 (24.5)
Western Europe	1779 (26.1)	1774 (26.1)
South America	270 (3.96)	264 (3.89)
Rest of World	943 (13.8)	946 (13.92)

Conduct of the study

Study TAAL was conducted in 725 centres in 30 countries around the world. The following changes were made in the conduct of the study after the start of the study: The definition of nonfatal periprocedural myocardial infarction (PPMI) within 48 hours after percutaneous coronary intervention was modified (protocol amendment A). The modified definition maintains the original definition and extends the definition of PPMI to include a CK-MB >5xULN on one sample if it is the last available sample and was drawn ≥12 hours after PCI. This change affected only the CEC adjudication of PPMI

within 48 hours of PCI. The criteria for investigator-identified nonfatal clinical MI that were also adjudicated by the CEC remained unchanged..

Overall, changes made during study conduct were not considered major.

Baseline data

Summary of baseline characteristics for subjects in study TAAL is presented below.

Characteristic	Prasugrel	Clopidogrel
Clinical Presentation n (%)	N=6813	N=6795
UA/NSTEMI	5044 (74.0)	5030 (74.0)
STEMI	1769 (25.9)	1765 (26.0)
STEMI ≤12 hours ^a	1203 (17.7)	1235 (18.2)
STEMI >12 hours ^a	564 (8.3)	530 (7.80)
Age (Years)	N=6813	N=6795
Overall Mean (SD)	60.9/11.2	60.9/11.4
≥75 Years n (%) ^a	901/13.2	908/13.4
Sex n (%)	N=6813	N=6795
Female	1705 (25.0)	1818 (26.8)
Region of Enrollment n (%)^a	N=6813	N=6795
North America	2164 (31.8)	2146 (31.6)
United States	2039 (29.9)	2020 (29.7)
Europe	3436 (50.4)	3439 (50.6)
Eastern Europe	1657 (24.3)	1665 (24.5)
Western Europe	1779 (26.1)	1774 (26.1)
South America	270 (3.96)	264 (3.89)
Rest of World	943 (13.8)	946 (13.92)
Body Weight (kg)	N=6722	N=6715
Mean (SD)	83.6 (16.8)	83.2 (16.9)
Creatinine Clearance	N=6699	N=6681
<60 ml/min	717 (10.7)	774 (11.6)
Index Procedure(s)	N=6813	N=6795
PCI	6715 (98.6)	6698 (98.6)
Multivessel PCI	967 (14.7)	946 (14.4)
CABG	25 (0.37)	23 (0.34)
Any Stent	6018 (95.7)	6004 (96.1)
Bare Metal Stent	3190 (51.0)	3185 (51.0)
Drug Eluting Stent	2860 (45.5)	2872 (46.0)
GPIIb/IIIa inhibitor use	3670 (53.9)	3733 (55.0)
Medical History n (%)^a	N=6813	N=6795
Diabetes Mellitus	1576 (23.1)	1570 (23.1)
Hypertension	4370 (64.1)	4371 (64.3)
Hypercholesterolemia	3790 (55.6)	3790 (55.8)
Prior MI	1226 (18.0)	1208 (17.8)
Prior PCI	904 (13.3)	926 (13.6)
Prior CABG	541 (7.94)	497 (7.3)
Prior TIA	94 (1.38)	117 (1.7)
Prior Stroke	181 (2.66)	160 (2.35)

Abbreviations: CABG = coronary artery bypass graft; MI = myocardial infarction; N = number of subjects randomly assigned; n = number of subjects in sub-category; NSTEMI = non-ST segment elevation myocardial infarction; PCI = percutaneous coronary intervention; SD = standard deviation; STEMI = ST-segment elevation myocardial infarction; TIA = transient ischemic attack; UA = unstable angina.

^a % is percent (rounded to nearest whole number) of number of subjects with non-missing values for category.

The majority of subjects were male and Caucasian. The mean age was 61 years and the mean weight was 83 kg. The subject characteristics were well balanced across the treatment groups; in UA/STEMI, STEMI, and all ACS populations. Exceptions of statistically significant differences were age and

diabetic treatment in the STEMI population, gender in the All ACS population, and the use of angiotensin-converting enzyme inhibitors (ACEI) in the UA/NSTEMI and the All ACS populations. The magnitude of the imbalances was small and these imbalances are not believed to affect the outcome of the study. It is of note that TIMI Risk Index score at baseline was identical in the all ACS population in the two treatment arms. There is a small difference - albeit statistically insignificant - in number of patients with a history of prior stroke between the two treatment arms.

Numbers analysed

The ITT population is defined as all randomized subjects except where otherwise specified. The Safety population is formed by all randomised patients who received at least one dose of the medication and had at least one contact with the investigator afterwards. Overall compliance with taking study drug was high (approximately 96%).

Outcomes and estimation

Study TAAL demonstrated that treatment with prasugrel, as compared with clopidogrel at the standard, approved dose, resulted in a statistically significant reduction in the rate of the primary efficacy endpoint (the composite of CV death, nonfatal MI, or nonfatal stroke at a median of 14.5 months follow-up). In addition, there was a statistically significant reduction in all pre-specified secondary efficacy endpoints. This was shown across the full spectrum of ACS with planned PCI. The primary and secondary efficacy endpoints were analyzed first in subjects with unstable angina and non-ST-segment elevation myocardial infarction (UA/NSTEMI), followed by an analysis of the same endpoints in all subjects in the All ACS population (UA/NSTEMI and ST-segment elevation myocardial infarction (STEMI)). The data were then also analysed in patients presenting with STEMI. For subjects presenting with UA/NSTEMI, the number and percentage of subjects reaching the primary composite endpoint were 469/5044 (9.30%) and 565/5030 (11.23%) for those randomized to prasugrel or clopidogrel, respectively, (HR=0.820 (95% CI 0.726-0.927)). For subjects presenting with STEMI, the number and percentage of subjects reaching the primary composite endpoint were 174/1769 (9.84%) and 216/1765 (12.24%) for those randomized to prasugrel or clopidogrel, respectively, (HR=0.793 (95% CI 0.649-0.968)). In the all ACS population, the number and percentage of subjects reaching the primary composite endpoint were 643/6813 (9.44%) and 781/6795 (11.49%) for those randomized to prasugrel or clopidogrel, respectively, (HR=0.812 (95% CI 0.732-0.902)). Thus, on average a relative risk reduction of approximately 20% was achieved. An absolute risk reduction of approximately 2% was observed.

Considering the individual components of the main endpoints significant reductions in the prasugrel group in the rates of ischemic events were observed. These differences were largely related to reduction in nonfatal MI (6.97% P vs 9.12% C, HR 0.757, $p < .001$) and all MI (7.12% P vs. 9.32% C, HR 0.757, $p < .001$). Positive results were evident within the first 24 hours following PCI, thus data seem to demonstrate a reduction in the early ischemic events such as peri-procedural MI. The risks of nonprocedural clinical MI were significantly reduced in the prasugrel group, as was the risk of new ST-elevation MI. However, this effect was not associated with a difference in the incidence of all cause death or CV death between treatment groups. It is of note that the higher level of platelet inhibition achieved relatively fast with the LD of prasugrel, leads to a reduction of the risk of thrombotic complications in the acute phase. Statistically significant differences in favour of prasugrel were also detected for all the planned secondary efficacy endpoints (see Methods, Objectives on page 35).

Study TAAL Primary Efficacy Endpoint and Components at Study End				
Event	Prasugrel	Clopidogrel	Hazard Ratio	p-Value^c
	n (%)^a	n (%)^a	(95% CI)^b	
UA/NSTEMI	N=5044	N=5030		
Primary End Point CV Death, Nonfatal MI, or Nonfatal Stroke	469 (9.30)	565 (11.23)	0.820 (0.726,0.927)	0.002
CV Death	90 (1.78)	92 (1.83)	0.979 (0.732,1.309)	0.885
Nonfatal MI	357 (7.08)	464 (9.22)	0.761 (0.663,0.873)	<0.001
Nonfatal Stroke	40 (0.79)	41 (0.82)	0.979 (0.633,1.513)	0.922
All Cause Death	130 (2.58)	121 (2.41)	1.076 (0.840,1.378)	0.563
All MI	366 (7.26)	476 (9.46)	0.760 (0.663,0.871)	<0.001
All Stroke	49 (0.97)	46 (0.91)	1.068 (0.714,1.597)	0.748
STEMI	N=1769	N=1765		
Primary End Point CV Death, Nonfatal MI, or Nonfatal Stroke	174 (9.84)	216 (12.24)	0.793 (0.649,0.968)	0.019
CV Death	43 (2.43)	58 (3.29)	0.738 (0.497,1.094)	0.129
Nonfatal MI	118 (6.67)	156 (8.84)	0.746 (0.588,0.948)	0.016
Nonfatal Stroke	21 (1.19)	19 (1.08)	1.097 (0.590,2.040)	0.770
All Cause Death	58 (3.28)	76 (4.31)	0.759 (0.539,1.068)	0.113
All MI	119 (6.73)	157 (8.90)	0.748 (0.589,0.949)	0.016
All Stroke	26 (1.47)	25 (1.42)	1.032 (0.596,1.787)	0.911
All ACS	N=6813	N=6795		
Primary End Point CV Death, Nonfatal MI, or Nonfatal Stroke	643 (9.44)	781 (11.49)	0.812 (0.732,0.902)	<.001
CV Death	133 (1.95)	150 (2.21)	0.886 (0.701,1.118)	0.307
Nonfatal MI	475 (6.97)	620 (9.12)	0.757 (0.672,0.853)	<0.001
Nonfatal Stroke	61 (0.90)	60 (0.88)	1.016 (0.712,1.451)	0.930
All Cause Death	188 (2.76)	197 (2.90)	0.953 (0.781,1.164)	0.639
All MI	485 (7.12)	633 (9.32)	0.757 (0.673,0.852)	<0.001
All Stroke	75 (1.10)	71 (1.04)	1.055 (0.763,1.460)	0.745

Abbreviations: ACS = acute coronary syndromes; CI = confidence interval; CV = cardiovascular; MI = myocardial infarction; N = number of randomly assigned subjects; n = number of subjects in sub-category; NSTEMI = non-ST-elevation myocardial infarction; STEMI = ST-segment elevation myocardial infarction; UA = unstable angina.

^aPercentage of randomly assigned subjects reaching the primary endpoint.

^bHazard ratio and a 95% CI used as an estimate of overall relative risk, prasugrel versus clopidogrel, over the course of the study.

^cTwo-sided p-values are based on Gehan-Wilcoxon test comparing event free survival distributions of prasugrel and clopidogrel for the composite primary endpoint. The individual components of the endpoints were tested using log-rank test. Clinical presentation, UA/NSTEMI versus STEMI, was used as a stratification factor in analysis involving All ACS subjects.

Efficacy was preserved across major pre-specified subgroups: sex, age (older or younger than 65 years), history of diabetes, type of stent employed, use of glycoprotein inhibitors, mono- or poly antithrombin use, dose of aspirin, renal function, geographical region. The treatment benefit was durable. The incidence of primary and secondary composite endpoints was statistically significantly lower in subjects treated with prasugrel compared to clopidogrel in the acute phase (within 3 days), the subacute phase (within 30 days), and in the long-term phase.

Secondary Outcome Events in Study TAAL – All ACS population				
Outcome Events	prasugrel + ASA (N=6813) %	Clopidogrel +ASA (N=6795) %	Hazard Ratio (95% CI)	p- value
CV death, nonfatal MI or nonfatal stroke through 90 days	6.8	8.4	0.797 (0.705,0.901)	<0.001
CV death, nonfatal MI or nonfatal stroke through 30 days	5.7	7.4	0.767 (0.672,0.876)	<0.001
CV Death, Nonfatal MI or urgent target vessel revascularisation (UTVR) through 90 days	6.9	8.7	0.794 (0.703,0.896)	<0.001
CV death, nonfatal MI, or UTVR through 30 days	5.9	7.4	0.784 (0.688,0.894)	<0.001
All cause death, nonfatal MI, or nonfatal stroke through study end	10.2	12.1	0.831 (0.751,0.919)	<0.001
CV death, nonfatal MI, nonfatal stroke or rehospitalisation for cardiac ischemic event through study end	11.7	13.8	0.838 (0.762,0.921)	<0.001
Definite or probable stent thrombosis through study end ^a	0.9	1.9	0.494 (0.361, 0.677)	<0.001

a N=6422 for EFIENT and N=6422 for clopidogrel.

Ancillary analyses

Elderly patients and patients with weight <60 kg

Analyses of the risk for TIMI bleeding by different weight and age cut-offs in study TAAL indicate that the odds ratio for bleeding with 10 mg prasugrel increases around the weight limit of less than 60 kg and age limit of greater than or equal to 75 years. The additional analyses presented suggest an increased bleeding risk associated with weight < 60 kg and age ≥ 75 years and the CHMP raised this as a major objection regarding the proposed reduced 5 mg MD in these two populations.. However, patients over 75 years of age weighing more than 60 kg did not seem to have an increased prasugrel exposure to the same extent as in patients weighing <60kg. This issue was also addressed in the oral explanation held at the CHMP. The results of the analysis conducted *via* a PK/PD model to evaluate the dose 5 mg in patients < 60 kg or ≥ 75 years of age need clinical confirmation and the Company is conducting such studies as part of the follow-up measures..

Comparison of 10-mg Prasugrel Exposure by Weight and Age Categories - Study TAAL

Group	N	Mean Age (years)	Mean Weight (kg)	G-Mean AUC (ng*hr/mL)	Ratio of Geometric Mean (90% CI) ^a
≥60kg and <75years	996	58	85	81.3	
<60kg and <75years	36	60	54	101.9	1.254 (1.105, 1.422)
<60kg and ≥75years	11	80	53	127.5	1.569 (1.252, 1.965)
≥60kg and ≥75years	110	79	78	94.5	1.163 (1.079, 1.253)

Abbreviations: CI = confidence interval; G-Mean = geometric least square mean; N = number of subjects in the specified subgroup.

^aversus ≥60 kg and <75 years

The question of the proposed reduction in the maintenance dose of prasugrel by one half in elderly patients (> 75 years) to reduce the risk of bleedings while not compromising the efficacy of the drug was discussed by the Scientific Advisory Group requested by the CHMP. This was also addressed at the oral explanation. Based on the increased risk of bleeding in patients ≥ 75 years of age treated with a 10 mg maintenance dose, a very strong wording in the SPC is implemented, stating that use in patients ≥75 years of age is generally not recommended and advising caution for the use of prasugrel in the elderly ≥75 years (i.e. individual benefit/risk evaluation and reduced maintenance dose of 5 mg). Although, the evidence for a 5 mg dose is based only on PK/PD analyses and no clinical data currently exist on the safety of this dose in the very elderly, it is believed that the treatment option in specifically selected and evaluated elderly patients at increased risk for ischemic events should be open after a careful, individual risk benefit evaluation.

In addition, reliable risk minimisation measures must be put in place and safety and efficacy data from clinical trials with this sub-population must be provided to the CHMP, as stated in the list of follow up measures. Adequate educational strategies prepared along with the scientific societies are to be put in place as requested by the CHMP as a condition for the safe and effective use of this medicinal product.

- Clinical efficacy results in special populations

In-stent thrombosis

Applying the ARC definitions (which included angiographic and clinical principles), there was a significant reduction in stent thrombosis in the prasugrel group including both reductions in early (<30 days) and late (≥ 30 days) stent thrombosis that was consistent in the 3 populations (UA/NSTEMI, STEMI, and All ACS). The RRR observed in both UA/NSTEMI and STEMI groups it is stated to be of nearly a 50%. A significant reduction in the rate of the incidence of the primary endpoint was found among patients receiving prasugrel in combination with bare-metal stents (9.37% P vs. 11.59% C) alone and in those receiving prasugrel in combination with at least one drug-eluting stent (8.67% P vs. 10.86% C). A lower incidence in the need of urgent target-vessel revascularization in the prasugrel group was also found.

Previous stroke/TIA

From the multivariate analysis the only risk factors differentially influencing the primary efficacy endpoint for prasugrel compared with clopidogrel were prior TIA or stroke and diabetes (see below). In particular, primary endpoint results in the All ACS population in those that had a prior history of TIA or stroke seem to favour clopidogrel. (prasugrel N 262 n 47 (17.94%) vs clopidogrel N 256 n 35 (13.67%) HR 1.375 CI 95% (0.886, 2.132) p= 0.153). Also, a higher incidence of nonfatal stroke and all stroke either hemorrhagic or non-hemorrhagic, when compared with clopidogrel (nonfatal stroke: 5.73% versus 0.78%, p-value =.002; all stroke: 6.49% versus 1.17%, p-value =.002) was observed. These patients with prior TIA or stroke have now been contraindicated to prasugrel.

Diabetes

For the diabetic population the incidence of the primary efficacy endpoint (All ACS, prasugrel N 1576 n 180 (11.42%) vs clopidogrel N 1570 n 248 (15.80%) HR 0.709 CI 95% (0.582, 0.854) p= 0.001) and each secondary efficacy composite endpoint was lower in subjects randomized to prasugrel compared to subjects randomized to clopidogrel in all 3 populations (UA/NSTEMI, STEMI, and All ACS). Recently published clinical results have suggested that subjects with diabetes may have greater

platelet reactivity and a lower antiplatelet response during clopidogrel treatment. In contrast, the current observations suggest that in subjects with stable CAD (study TABR and TABL), prasugrel treatment provided consistent levels of platelet inhibition in those with and without diabetes. It is proposed that more potent platelet inhibition with prasugrel may result in improved clinical outcomes in ACS subjects with diabetes.

- Discussion on clinical efficacy

Regarding clinical relevance and general interpretation of the results in the context of current evidence, study TAAL could be large enough to address separately the thienopyridine-mediated platelet inhibition in the two major presentation forms of acute coronary syndrome (that is, UA/NSTEMI and STEMI). The median time from onset of qualifying symptoms to randomization in study TAAL in subjects presenting with UA/NSTEMI was 28.9 and 29.0 hours for patients randomized to prasugrel or clopidogrel treatment, respectively. Upper quartiles were 48.6 and 49.0 hours, respectively. In study TAAL, all patients with UA/STEMI were randomized after coronary pathoanatomy was known, i.e. after coronary angiography. The strategy of administering the thienopyridine LD once coronary anatomy is known appears to be preferred because of concerns about surgical bleeding in patients treated with clopidogrel that subsequently undergo CABG surgery. It is acknowledged that the optimum timing of platelet inhibition with a thienopyridine has been debated in recent years. The benefits of the early administration of clopidogrel before PCI does not come from randomised clinical trials primarily aimed to this end, but from post-hoc subgroup analyses and observational studies.

The primary objective of study TAAL was to test the hypothesis that prasugrel co-administered with aspirin was superior to clopidogrel co-administered with aspirin in the treatment of subjects with acute coronary syndromes (ACS) who were to undergo percutaneous coronary intervention (PCI), as measured by a reduction in the composite endpoint of cardiovascular (CV) death, nonfatal myocardial infarction (MI), or nonfatal stroke at a median follow-up of at least 12 months. It was pre-specified that the primary endpoint was analyzed first in subjects with unstable angina and non-ST-segment elevation myocardial infarction. Efficacy superiority of prasugrel has been fully demonstrated for the primary and all secondary endpoints. As mentioned earlier, the timing of prasugrel LD treatment in study TAAL deviated from the present treatment guidelines and this initially raised objection was addressed by presenting several lines of evidence from study TAAL, all of which suggested that the timing of clopidogrel LD did not substantially influence the overall efficacy (or safety) of prasugrel observed in this trial. It was noted that for subjects treated with GPIIb/IIIa inhibitors, there was no evidence that the relative benefit for prasugrel versus clopidogrel was reduced or that there was an excess need for bail out GPIIb/IIIa inhibitor use during PCI in those patients randomised to clopidogrel in the TAAL study. This observation could indirectly indicate that the timing of study drug LD did not substantially influence overall efficacy. Furthermore, when study drug LD is administered before or during PCI, both clopidogrel and prasugrel would be at their near-maximal levels of platelet inhibition achievable by the LD in the early hours following the PCI. This claim is well supported by pharmacokinetic data and *ex vivo* platelet inhibitory activity for prasugrel and clopidogrel, and supports that the timing of study drug LD did not substantially influence the overall efficacy. Stronger evidence for this position originates in the subgroup analysis of patients pre-treated with thienopyridine. In study TAAL, if coronary anatomy was determined or primary PCI for STEMI (≤ 12 hours) was planned, pre-treatment with study drug was allowed for up to 24 hours before PCI. The percentage of subjects in this pre-treated subgroup reaching the composite endpoint of CV death, nonfatal MI, or nonfatal stroke from randomisation through study end was 9.94 and 11.29, respectively, for subjects pre-treated with prasugrel or clopidogrel. Although not statistically significant for this subgroup, the difference rather strongly favours the notion that the timing of study drug LD to a large extent did not influence overall efficacy. Additional examination of the subgroup data shows that the predominant benefit of being randomised to prasugrel treatment is not evident in the reduction in peri-procedural MI, a time when prasugrel would presumably have the greatest early advantage, but rather in the reduction in subsequent clinical MI. It is acknowledged that this observation also supports the position that timing of study drug LD is not crucial to the overall efficacy. Finally, the analysis of long-term clinical benefits in Study TAAL confirms the lack of influence of timing of study drug on efficacy. Considering all of these lines of evidence, it is unlikely

that timing of study drug LD had major importance to the overall efficacy demonstrated in Study TAAL.

Regarding the subjects presenting with STEMI, the number and percentage of subjects reaching the primary composite endpoint were 174/1769 (9.84%) and 216/1765 (12.24%) for those randomized to prasugrel or clopidogrel, respectively, (HR=0.793 (95% CI 0.649-0.968)). The treatment benefit was durable; at 3 days, at 30 days, and at study end. Regarding possible effects of prior fibrinolytic treatment, the percentage of subjects reaching the composite endpoint was 6.4% and 8.7% (randomized to prasugrel or clopidogrel, respectively) if fibrinolytic therapy was used before PCI. The corresponding percentages if fibrinolytic therapy was not used were 10.2% and 12.7%. There was no significant statistical interaction between the treatment benefit observed with prasugrel and prior treatment with a fibrinolytic agent in those presenting with STEMI. It is therefore unlikely that the efficacy benefit with prasugrel in subjects presenting with STEMI was influenced by the administration of a fibrinolytic agent prior to PCI. There was a lower incidence of the primary composite endpoint in subjects randomized to prasugrel compared to clopidogrel in the STEMI population undergoing primary (≤ 12 hours) PCI (10.06 % versus 11.50%; HR=0.872; p=.266). In the STEMI population undergoing delayed (> 12 hrs) PCI the corresponding values were 9.40 % versus 13.96%; HR=0.649; p=.015. Initially, these data suggest that patients presenting with STEMI late after symptom onset benefit from prasugrel treatment in particular. However, these patients were in principle handled like patients with UA/NSTEMI, i.e. they were randomised and received study treatment after diagnostic coronary angiography. In addition, the description of the primary endpoint was simplified in the wording of the indication to increase the clarity, but data on each of the individual components of the primary endpoint is retained in the SPC in a relevant section.

For some patients at special risk (very elderly ≥ 75 years, patients weighing < 60 kg) dose reduction is suggested. After an LD of 60 mg, an MD of 5 mg once daily is recommended, but these patients were not adequately studied with a 5 mg maintenance dose. These considerations formed the basis of a major objection raised by the CHMP. New safety/efficacy analyses based on the active metabolite exposure and PK/PD simulations were presented in the CHMP oral explanation and were conducted in order to support the proposed reduction to a 5 mg prasugrel MD in subjects < 60 kg or ≥ 75 years of age. The reduction of the dose is proposed for the following reasons:

- higher exposure to the prasugrel active metabolite in this sub-population associated with an increased risk of bleeding
- bleeding in subjects < 60 kg or ≥ 75 years was predominantly confined to subjects with the highest exposure
- subjects < 60 kg or ≥ 75 years of age with lower exposure to the active metabolite had similar risk of bleeding as subjects ≥ 60 kg or < 75 years of age
- based on the results of a predictive model, reducing the prasugrel MD to 5 mg in subjects < 60 kg or ≥ 75 years of age produces similar exposure as was observed in the lowest quartile exposure in the overall population in study TAAL on the prasugrel 10 mg MD, a quartile of exposure where efficacy was maintained and the risk of bleeding was lowered.

In addition, two dedicated studies aimed to compare the PK, PD, safety, and tolerability of prasugrel in subjects < 60 kg or ≥ 75 years treated with a MD of either prasugrel 5-mg, prasugrel 10-mg, or clopidogrel 75-mg will be conducted as part of the follow up measures. Further a post-authorisation study will be performed to assess the benefit/risk of prasugrel used in real life setting. Results from the study H7T-MC-TABY (TABY) with 10,000 randomised subject assessing the efficacy and safety of prasugrel compared to clopidogrel in medically managed subjects with ACS who have experienced a recent UA/NSTEMI event will be made available to the CHMP and this commitment is part of the follow up measures. The CHMP accepted the proposed dose reduction in patients weighing < 60 kg. Although, the dose-reduction in the very elderly ≥ 75 years and the benefit/risk with the 5 mg MD is not fully clinically supported at present, it is believed that the treatment option in specifically selected and evaluated elderly patients at increased risk for ischemic events should be open after a careful, individual risk benefit evaluation.

Clinical safety

Introduction

The clinical safety evaluation of prasugrel is primarily based on the pre-specified primary database from the pivotal TAAL study. It includes data from 13457 treated subjects with acute coronary syndromes (ACS) undergoing percutaneous coronary intervention (PCI) who were treated with prasugrel (6741 subjects) or clopidogrel (6716 subjects), co-administered with aspirin for up to 15 months. These patients have been exposed to the proposed prasugrel dosing regimen (60-mg loading dose [LD]/10-mg maintenance dose [MD]). The secondary safety database includes data from the 4 smaller studies; TAAD, TAAH, TABL, TABR grouped into "All but TAAL (ABT)", and Study TAAL limited to the first 30 days after first dose of study drug, "TAAL-30". The tertiary safety database comprises the safety data from clinical pharmacology studies. Two major populations contributed to the All ACS population:

- UA/NSTEMI (clinical presentations being UA or NSTEMI within 72 hours)
- STEMI (clinical presentations being STEMI \leq 12 hours since symptoms onset or STEMI >12 hours since symptoms onset)

The number of subjects with STEMI was capped at 3534 subjects. The majority of subjects in the All ACS population were male (74%) and Caucasian (92%). The mean age was 61 years and the mean weight was 83 kg. The geographic region of origin was Europe in approximately 50% and North America in 32%.

- Patient exposure

A total of 8656 subjects (6741 from the primary safety database, 940 from the secondary safety database and 975 from the tertiary safety database) have been exposed to at least 1 dose of prasugrel across all completed clinical and clinical pharmacology studies. The overall exposure to prasugrel in the primary safety database was 6483 subject-years. More than half of the subjects treated with prasugrel were exposed for more than 1 year. For the primary safety database "while at risk" was defined as the period from first study drug administration through 7 days after permanent study drug discontinuation (the termination visit) or through 464 days after randomisation, whichever came first.

- Adverse events

In the primary safety database, treatment-emergent adverse events (TEAEs) were reported in 80.34% of prasugrel treated patients and 80.02% of clopidogrel treated patients. Clinically significant AEs were also pre-specified apart from CEC adjudicated bleeding endpoints, and included AEs of particular interest (thrombocytopenia, thrombotic thrombocytopenic purpura [TTP], neutropenia, leucopenia, pancytopenia, torsades de pointes/QT prolongation, allergic reactions, and abnormal hepatic function). No statistically significant difference between treatments was observed for these clinically significant TEAEs (not either for TEAEs and SAEs) when the analysis was limited to the first 3 days (prasugrel 3.16%, clopidogrel 2.75%) or the first 30 days (prasugrel 5.34%, clopidogrel 5.0%). The majority of drug related adverse events were related to bleeding and the risk was higher with prasugrel than with clopidogrel.

Hemorrhagic AEs occurred with a statistically significant higher incidence in the prasugrel treated patients compared to clopidogrel, 29.70% vs 22.04% ($p < 0.001$). Both CEC- adjudicated non-CABG-related TIMI Major Bleeding, TIMI Major or Minor Bleeding and TIMI Major, Minor, or Minimal Bleeding were statistically significantly increased in prasugrel vs. clopidogrel patients (2.17% vs. 1.65%, 4.49% vs. 3.44%, and 10.86% vs. 7.86%, respectively). Though numbers of patients undergoing CABG were small (213 in the prasugrel group, 224 in the clopidogrel group), it appeared that the risk of CABG related TIMI Major or Minor Bleeding was approximately tripled in the prasugrel arm (30 patients [14.08%] vs. 10 patients [4.46%], $p < 0.001$). The overall distribution of hemorrhagic TEAEs and the higher incidence of hemorrhagic TEAEs in prasugrel and clopidogrel treated patients, respectively, was comparable between the UA/NSTEMI subgroup (prasugrel 29.95%, clopidogrel 22.21%), and the STEMI subgroup (prasugrel 28.97%, clopidogrel 21.54%). Nevertheless, although the number of STEMI patients receiving fibrinolytic treatment was small, the bleeding events were comparable between STEMI patients who received fibrinolytic treatment and the patients who did not receive fibrinolytic treatment. In addition, the efficacy benefit seen with prasugrel treatment in

the STEMI population, was not outweighed by bleeding complications in STEMI sub-population of patients managed with delayed PCI. In order of descending frequency, contusion, haematoma, epistaxis, ecchymosis, vessel puncture site haematoma, puncture site haemorrhage, haematuria, and GI haemorrhage were the common ($\geq 1\%$) hemorrhagic ADRs associated with prasugrel therapy. In the secondary safety database, the overall incidence of hemorrhagic events was higher in ABT versus TAAL-30 for both treatment groups (ABT: prasugrel 35.74%, clopidogrel 20.66%; TAAL-30: prasugrel 18.29%, clopidogrel 14.34%). This was primarily due to the incidence of catheter site haematoma, catheter site haemorrhage, contusion, and epistaxis.

Non-haemorrhagic AEs occurred with a similar frequency in the two treatment groups (77.33% with prasugrel and 77.86% with clopidogrel); statistically significant differences were seen for coronary revascularisation, fatigue, MI, constipation, musculoskeletal pain, cardiac failure (more frequent with clopidogrel) and for pyrexia and tendency to bruise (more frequent with prasugrel). The incidence of infections was similar between the two treatment groups. It is believed that the observed differences in the incidence of pyrexia are in part due to the higher incidence of bleeding in subjects treated with prasugrel, who more frequently received transfusions. Additionally, the causality relationship between rash and prasugrel could not be excluded. The issues have been adequately addressed in the RMP as a potential risk and pharmacovigilance measures will be implemented.

Colon cancer was an uncommon TEAE (0.17% with prasugrel, 0.03% with clopidogrel) that occurred with a statistically significant higher incidence ($p=0.013$) in subjects treated with prasugrel. Of the 19 reports from the prasugrel group, 10 were diagnosed as a result of a diagnostic procedure following a gastrointestinal bleeding. On the basis of these findings, it was concluded that colon cancer was diagnosed more often in subjects treated with prasugrel due to a higher rate of bleeding associated with this therapy.

- Serious adverse event/deaths/other significant events

Deaths

The overall incidence of all-cause deaths was similar between the treatment groups in the primary database (clopidogrel 2.90%, prasugrel 2.76%). The majority of deaths were cardiovascular deaths (prasugrel 1.95% vs clopidogrel 2.21%). In the UA/NSTEMI subpopulation, a numerically higher overall mortality was observed in the prasugrel treatment group compared to clopidogrel. However, the explanation is acceptable that the observed numerical increase in overall mortality in prasugrel-treated patients with UA/NSTEMI (9 more deaths) cannot be disentangled from the recognised increased risk of bleeding associated with prasugrel. Elderly patients constitute an especially sensitive population regarding bleeding risk, and explain most, if not all, of the numerical differences in mortality observed in the UA/NSTEMI population.

There was a higher incidence of deaths due to haemorrhage in prasugrel treated patients (both ICH (prasugrel 9 (0.13%) vs clopidogrel 5 (0.07%) and non-ICH (prasugrel 9 (0.13%) vs clopidogrel 1 (0.01%)) in the All ACS population (see below). The SPC was revised to state the increased risk of major, life-threatening and fatal bleeding associated with the use of prasugrel as compared to clopidogrel in the UA/NSTEMI and All ACS populations.

Summary of Deaths in Study TAAL; All Randomized All ACS Subjects

Deaths All ACS Population	Prasugrel N=6813 n (%) ^a	Clopidogrel N=6795 n (%) ^a	Total N=13608 n (%) ^a
Deaths during study period ^b	188	197	385
Deaths in treated subjects	181	186	367
Deaths in subjects not treated with study drug	7	11	18
Deaths outside of the study period ^b	3	0	3
All Total Deaths ^c	191	197	388
Clinical Endpoints Committee Adjudicated Deaths			
All Cause Death ^c	188 (2.76)	197 (2.90)	385 (2.83)
Cardiovascular Death	133 (1.95)	150 (2.21)	283 (2.08)
Atherosclerotic Vascular Disease ^d	0	3 (0.04)	3 (.0002)
CHF/Cardiogenic Shock	31 (0.46)	30 (0.44)	61 (0.45)
Directly related to revascularization ^e	15 (0.22)	16 (0.24)	31 (0.23)
Dysrhythmia	4 (0.06)	7 (0.10)	11 (0.08)
Pulmonary Embolism	3 (0.04)	0	3 (0.02)
Myocardial Infarction	24 (0.35)	36 (0.53)	60 (0.44)
Sudden or Unwitnessed	36 (0.53)	42 (0.62)	78 (0.57)
Intracranial Hemorrhage	9 (0.13)	5 (0.07)	14 (0.10)
Non-Hemorrhagic Stroke	5 (0.07)	6 (0.09)	11 (0.08)
Other Cardiovascular	6 (0.09)	5 (0.07)	11 (0.08)
Non-Cardiovascular Death	55 (0.81)	47 (0.69)	102 (0.75)
Accidental/Trauma	4 (0.06)	4 (0.06)	8 (0.06)
Nonintracranial Hemorrhage	9 (0.13)	1 (0.01)	10 (0.07)
Infection	11 (0.16)	10 (0.15)	21 (0.16)
Malignancy	21 (0.31)	17 (0.25)	38 (0.28)
Suicide	3 (0.04)	2 (0.03)	5 (0.04)
Other Non-Cardiovascular	7 (0.10)	13 (0.19)	20 (0.15)

Abbreviations: ACS = acute coronary syndromes; CHF = coronary heart failure; PCI = percutaneous coronary intervention; UA = unstable angina.

^a % is percentage of randomized subjects.

^b Study period = from randomization through a subject's study termination or 464 days from randomization, whichever was earlier.

^c There are a total of 388 deaths during the study, with 3 deaths occurring outside the study period, which were listed in the row of "Deaths outside of study period." Therefore, "All total deaths" and "All Cause deaths" differ by 3 subjects.

^d Atherosclerotic vascular disease excludes deaths from coronary vascular disease.

^e Death is directly related to hemorrhagic or non-hemorrhagic complications of revascularization (CABG or PCI).

Fatal haemorrhages

Overall, in the All ACS population fatal hemorrhagic events (including CABG-related and Non-CABG-related bleeding events) occurred in 24 subjects (0.36%) in the prasugrel treatment group and 6 subjects (0.09%) in the clopidogrel treatment group. The majority (21/24 deaths in prasugrel patients, 5/6 deaths in clopidogrel patients) were non CABG related TIMI major bleedings during the at risk period. Non CABG-related spontaneous intracranial and gastrointestinal (GI) bleedings were predominant (prasugrel: spontaneous fatal bleedings in 16 patients, hereof 8 intracranial and 6 GI bleedings; clopidogrel: spontaneous fatal bleedings in 4 patients, 4 intracranial and 1 GI bleeding). Non-CABG related instrumented fatal bleedings were only seen in the prasugrel group (4 patients). The incidence of fatal bleedings was also statistically significant in the UA/NSTEMI prasugrel group. The same pattern was observed in the STEMI population but due to insufficient data the statistics could not be evaluated (prasugrel: 7 patients, 0.40%, clopidogrel 2 patients 0.12%). There were no fatal hemorrhagic events in the secondary and tertiary databases.

SAEs

Serious adverse events occurred in 24.70% of prasugrel and 24.26% of clopidogrel treated patients in the primary database. The incidence of non-hemorrhagic SAEs was similar in the prasugrel (22.48%) and the clopidogrel (22.80%) group. Most frequent were non-cardiac chest pain, coronary artery re-

stenosis, chest pain and angina pectoris. Three non-haemorrhagic SAEs that occurred differentially among treatment groups were the higher incidence in the prasugrel group of colon cancer, hypotension, and respiratory failure (which was finally related to the simultaneous occurrence of blood loss). The incidence of hemorrhagic SAEs in the All ACS population while at risk was statistically significantly higher in subjects treated with prasugrel when compared to subjects treated with clopidogrel. Likewise, in the UA/STEMI group the incidence of hemorrhagic SAEs with prasugrel was statistically significantly higher (prasugrel 6.08% vs clopidogrel 4.06%). In the STEMI population the incidence was numerically higher though, not statistically different (5.34% vs. 4.26%). Non-CABG related TIMI Major Bleedings (including life threatening and fatal bleedings), TIMI Major or Minor, and TIMI Major, Minor, or Minimal Bleedings were all significantly higher in the prasugrel treated subjects compared to the clopidogrel treated subjects in the All ACS population.

Incidence of Bleeding Events—Clinical Events Committee Adjudicated – Primary Safety Database (Study TAAL) All ACS

Bleeding Events ^a	Prasugrel (N=6813) n (%)	Clopidogrel (N=6795) n (%)	Total (N=13608) n (%)	Hazard Ratio (95% CI) ^b	p- Value ^c
All ACS	6741	6716	13457	NE	NE
Non-CABG-related					
TIMI Major	146 (2.17)	111 (1.65)	257 (1.91)	1.315 (1.028, 1.683)	.029
Life-Threatening	85 (1.26)	56 (0.83)	141 (1.05)	1.517 (1.083, 2.126)	.015
Fatal	21 (0.31)	5 (0.07)	26 (0.19)	4.191 (1.580, 11.113)	.002
Symptomatic ICH	19 (0.28)	17 (0.25)	36 (0.27)	1.119 (0.582, 2.152)	.736
IV Inotrope Required	21 (0.31)	8 (0.12)	29 (0.22)	2.617 (1.159, 5.908)	.016
Surgery Required	19 (0.28)	19 (0.28)	38 (0.28)	0.998 (0.528, 1.885)	.995
Transfusion of ≥4 Units	45 (0.67)	30 (0.45)	75 (0.56)	1.499 (0.945, 2.379)	.084
Instrumented	45 (0.67)	38 (0.57)	83 (0.62)	1.182 (0.767, 1.820)	.447
Spontaneous	92 (1.36)	61 (0.91)	153 (1.14)	1.508 (1.091, 2.085)	.012
TIMI Minor	164 (2.43)	125 (1.86)	289 (2.15)	NE	NE
TIMI Major or TIMI Minor	303 (4.49)	231 (3.44)	534 (3.97)	1.314 (1.107, 1.559)	.002
TIMI Minimal	460 (6.82)	314 (4.68)	774 (5.75)	NE	NE
TIMI Major, Minor, or Min	732 (10.86)	528 (7.86)	1260 (9.36)	1.400 (1.252, 1.566)	<.001
Any Transfusion Required ^d	244 (3.62)	182 (2.71)	426 (3.17)	1.34 (1.11, 1.63)	.003
CABG-related	(N=213)	(N=224)	(N=437)		
TIMI Major or Minor	30 (14.08)	10 (4.46)	40 (9.15)	3.587 (1.702, 7.557) ^e	<.001
Fatal	2 (0.94)	0	2 (0.46)	NE	NE

Abbreviations: ACS = acute coronary syndromes; CABG = coronary artery bypass graft surgery; CI = confidence interval; HR = hazard ratio; ICH = intracranial hemorrhage; IV = intravenous; Min = minimal; N = number of subjects in the specified subgroup; n = number of subjects within the specified subgroup reaching the endpoint; NE = not evaluated due to insufficient sample size; NSTEMI = non-ST-segment elevation myocardial infarction; STEMI = ST-segment elevation myocardial infarction; TIMI = Thrombolysis in Myocardial Infarction; UA = unstable angina.

a Subjects experiencing multiple bleeding events may be included in more than one category.

b HR and two-sided 95% CI derived using Cox proportional hazards model.

c Two-sided log-rank p-value based on time to first event analysis compares the event free survival distributions for prasugrel and clopidogrel. Clinical presentation, UA/NSTEMI versus STEMI, was used as a stratification factor in analyses of All ACS subjects.

d Bleeding requiring any transfusion (whole- or packed-blood).

e Odds ratio is based on the frequency procedure. Two-sided p-values are based on Cochran-Mantel-Haenszel general association test with clinical presentation as a blocking factor in All ACS.

In the All ACS population, the significantly higher incidence of TIMI Major bleedings in subjects treated with prasugrel was related to higher rates of GI bleeding (prasugrel 0.93% vs. clopidogrel 0.64%), surgical site bleeding (0.15% vs. 0.01%), and bleeding at other sites not pre-specified or unknown (0.13% vs. 0.01%). A higher incidence of retroperitoneal bleeding was also observed in subjects treated with prasugrel (0.21% vs. 0.12%). Intracranial hemorrhages and puncture site bleeding events were similar between treatment groups (0.28% vs. 0.25% and 0.42% vs. 0.45%, respectively).

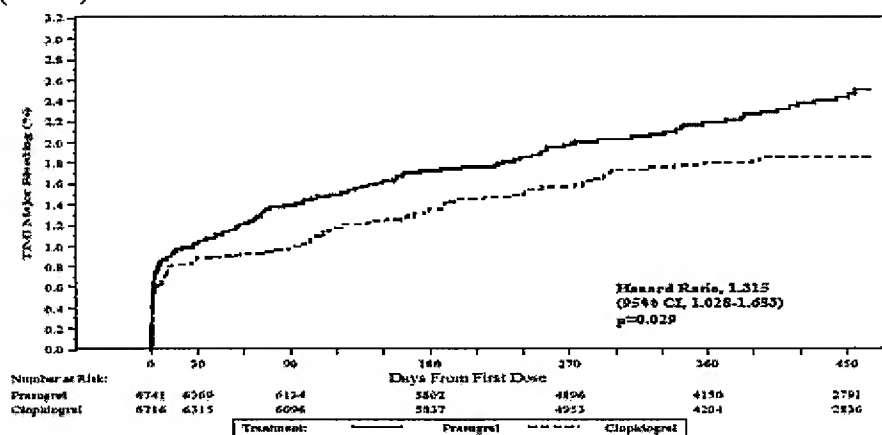
Non-CABG-related TIMI Major and TIMI Life-Threatening bleeding events through 3 days after the first dose of study: The incidence in the All ACS population was numerically higher in subjects treated with prasugrel compared to clopidogrel (prasugrel 0.74% vs. clopidogrel 0.61% for TIMI major and 0.43% vs. 0.31% for Life threatening bleeding), primarily related to a numerically higher incidence of GI bleeding which was reflected by a higher incidence of spontaneous bleeding events. Likewise, in the UA/NSTEMI population, there were numerically more spontaneous and instrumented, bleeding

events in subjects treated with prasugrel compared to clopidogrel through 3 days. In the STEMI population there were no relevant differences between the 2 treatment groups.

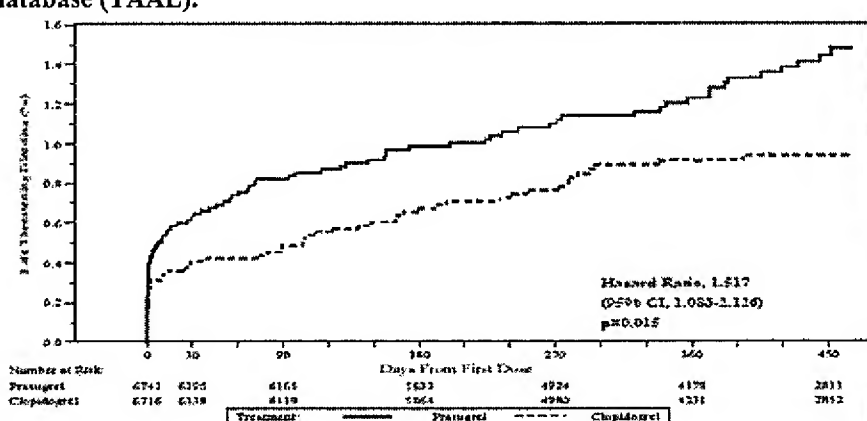
Non-CABG-related TIMI Major and TIMI Life-Threatening bleeding events beyond 3 days after the first dose of study: The incidence was statistically significantly higher in subjects in the All ACS population treated with prasugrel compared to clopidogrel (1.45% vs. 1.05% for TIMI major, 0.84% vs. 0.53% for TIMI life threatening bleeding), primarily driven by a numerically higher incidence of GI bleeding (0.78% vs 0.59%), surgical site bleeding (0.10% vs 0.02%), and bleeding at sites not pre-specified or unknown (0.10% vs 0%). A higher numerical incidence of retroperitoneal bleeding (0.09% vs 0.03%) and surgical site bleeding events (0.10% vs. 0.02%) was observed with prasugrel compared to clopidogrel, which resulted in a statistically significant higher incidence of spontaneous bleeding events (1.14% vs 0.78%) and a numerically higher incidence of instrumented bleeding events (0.19% vs 0.12%) with prasugrel compared to clopidogrel. For the subcategory of TIMI Life-Threatening bleeding, subjects treated with prasugrel had a statistically significant higher incidence of fatal bleeding (0.24% vs 0.06%) and a numerically significant higher incidence of bleeding requiring intravenous inotropic medication in addition to multiple transfusion units. A higher incidence in Non-CABG-related TIMI Life-Threatening bleeding events was observed in the UA/NSTEMI population, but not in the STEMI population.

The time course of TIMI major bleeding events and its subgroup Life threatening bleedings is shown in the following figures.

Kaplan-Meier estimates of the incidence of Non-CABG related TIMI major bleeding events while at risk—CEC adjudicated for all treated All ACS subjects in primary safety database (TAAL).



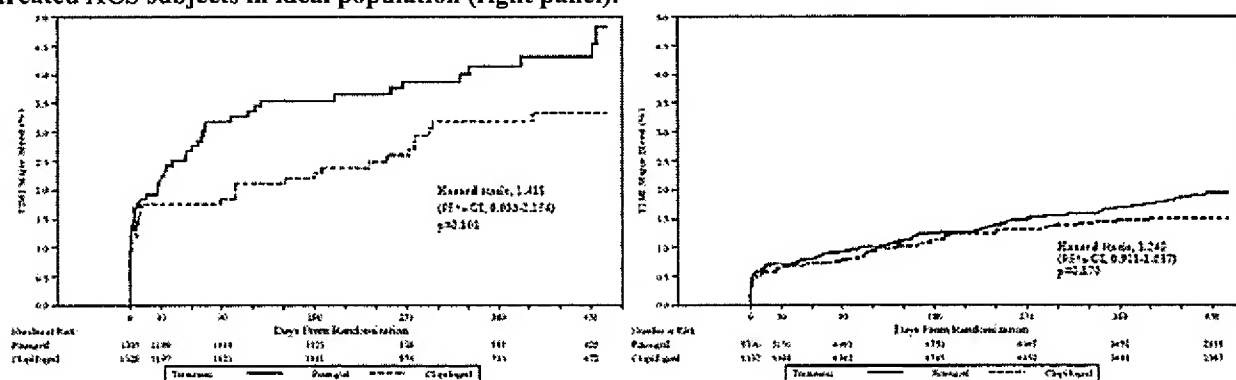
Kaplan-Meier estimates of the incidence of Non-CABG-related TIMI life-threatening bleeding events while at risk— CEC adjudicated for all treated All ACS subjects in primary safety database (TAAL).



The Kaplan Meier curves separated early for Non-CABG-related TIMI Major and TIMI Life-Threatening bleeding events while at risk, favouring clopidogrel. Another separation is seen beyond 1 year and potentially also at around 30 days. Multivariate analyses identified independent risk factors for increased occurrence of Non-CABG-related TIMI Major bleeding. These were prasugrel treatment, weight < 60 kg, age \geq 75 years, history of hypertension, history of prior TIA or stroke, and use of GPIIb/IIIa inhibitor (from symptom onset through 3 days after randomization).

Further analyses which demonstrated that mainly patients at risk (patients with a prior history of TIA/stroke, patients with body weight < 60 kg and patients \geq 75 years of age) are responsible for the unfavourable safety profile of prasugrel, were provided during the evaluation. The figures below display the Kaplan Meier curves for Non-CABG related TIMI Major bleeding events in the “ideal population” (excluding subjects with a history of TIA or stroke, subjects \geq 75 years of age taking a MD of 10 mg/day, or subjects <60 kg taking a MD of 10 mg/day) and in the non-ideal population (subjects with history of TIA or stroke, \geq 75 years of age or weighing <60 kg). The difference between prasugrel and clopidogrel between 30 and 90 days, is explained by the higher bleeding risk in subjects in the non-ideal population. However, major bleedings also appear to accrue beyond 1 year. The CHMP therefore requested to limit the treatment duration with prasugrel to 12 months, consistent with the existing treatment duration recommendation for clopidogrel in this clinical setting.

Kaplan-Meier estimates of the incidence of non-CABG-related TIMI Major bleeding events while at risk – CEC adjudicated treated ACS subjects in non-ideal population (left panel) and treated ACS subjects in ideal population (right panel).



Considering the ideal population only, the rate of Non-CABG-related TIMI Major and Life-Threatening bleeding in prasugrel treated subjects was numerically, but not statistically higher compared to clopidogrel-treated subjects. The number of fatal bleeding events beyond 3 days of treatment was 7 in prasugrel-treated subjects versus 2 in clopidogrel-treated subjects, as compared to 21 versus 5 in the All ACS population for the entire treatment duration ("at risk period"). Three of the 7 fatal bleeding events in prasugrel-treated subjects resulted from procedural complications, while 4 were fatal ICH (1 traumatic). Both clopidogrel fatal events were spontaneous ICH. Consequently, the number of spontaneous fatal bleeding events beyond 3 days during MD was similar between treatment groups. During the first 3 days, CABG and non-CABG related fatal bleedings were as follows: one fatal bleeding occurred in the clopidogrel group and 6 fatal bleedings (3 each from UA/NSTEMI and STEMI) occurred in the prasugrel group. Four of these patients belonged to the ideal population, and 3 of these had an instrumented or traumatic bleeding. Major bleeding may increase the short-term risk of ischemic events and the risk of death. This was evident in study TAAL. In addition, it was shown that for the All ACS population and both treatment groups together, there was no statistically significant difference between treatment groups for the risk of ischemic events (CV death, CV death/MI, CV death/MI/stroke) beyond and within 30 days in patients having experienced no major bleed versus patients having experienced a major bleed, although the risk was numerically higher for those with major bleed. A comparison of all-cause deaths within and beyond 30 days by treatment group and clinical presentation (UA/NSTEMI, STEMI) shows that mortality within 30 days is roughly comparable for prasugrel and clopidogrel (for the STEMI population even numerically slightly more favourable for prasugrel) if patients did not have a TIMI major bleeding. However, all cause death in patients with a TIMI major bleeding and treated with prasugrel was higher when compared to patients treated with clopidogrel (all ACS population 15.6% vs. 10.9% ($p=0.06$), similarly in the UA/NSTEMI and STEMI subgroups). Analysis of the "ideal population" shows that all cause death is still numerically higher for prasugrel compared to clopidogrel but the difference is not statistically significant (all ACS population 9.4% vs 7.3%, $p=0.52$). These deaths are predominantly attributable to fatal bleedings and the increased bleeding risk with prasugrel is clearly stated in the SPC. Beyond 30 days, mortality is comparable for prasugrel and for clopidogrel treated patients not having had a major bleed. For patients with major bleed, events are too few to draw firm conclusions. .

- **Laboratory findings**

The safety data suggests that prasugrel therapy is not associated with clinically significant thrombocytopenia, neutropenia, or leucopenia. The number of subjects with normal Hct, Hgb, or RBC at baseline, and abnormally low values at any time post-baseline, was statistically significantly higher in subjects treated with prasugrel compared to clopidogrel and probably reflects the higher incidence of bleeding events. Hepatotoxicity of prasugrel is not suggested by the laboratory data.

- **Safety in special populations**

Renal impairment: In total, 707 patients in the prasugrel group and 769 in the clopidogrel group had a creatinine clearance ≤ 60 ml/min (measured by Cockcroft-Gault formula). For both treatments (prasugrel vs clopidogrel) a higher incidence of TIMI major or minor bleeding events was observed in patients with creatinine clearance ≤ 60 ml/min compared to patients with normal renal function (9.48% and 6.76%, $p=0.052$ vs 3.89% and 2.98%). The same tendency was observed for life threatening bleeding events (2.26% and 1.04%, $p=0.059$ vs. 1.09% and 0.78%). Multivariate analysis did not identify renal impairment as expressed by creatinine clearance as a predictor of higher bleeding risk. More than half of patients with creatinine clearance <30 ml/min were also very elderly patients. When analysing bleedings excluding these patients >75 years, the bleeding risk was still elevated but not significantly different between prasugrel and clopidogrel.

Hepatic impairment: Active metabolite exposures in subjects with moderate hepatic impairment and in healthy subjects are comparable. Patients with severe hepatic impairment were excluded from TAAL and a contraindication for these patients is included in the SPC. There were only a limited number of patients with less severe forms of hepatic impairment included in TAAL. Although safety did not seem to be compromised in these patients, safety conclusions must be drawn cautiously.

Age: In total, 901 subjects in the prasugrel group and 908 subjects in the clopidogrel group were ≥ 75 years. In both treatment groups, twice as many subjects ≥ 75 years experienced Non-CABG-related

TIMI major or minor- (prasugrel 8.98%, clopidogrel 6.94%) as well as life-threatening bleeding events (prasugrel 2.58%, clopidogrel 1.57%) compared to patients below the age of 75 years. This was observed for both treatment groups. Furthermore, for prasugrel-treated patients ≥ 75 years (UA/NSTEMI as well as All ACS), twice as many experienced any stroke compared to clopidogrel treatment (2.89% vs 1.43%). A statistically significant difference was seen on the incidence of fatal bleeding in favour of clopidogrel. Use of prasugrel in this patient population is generally not recommended. If prescribed after a careful individual risk/benefit evaluation, a lower MD of prasugrel 5 mg should be used (please refer to section on Clinical efficacy). Further information on the 5-mg dose in this population will be obtained from future or ongoing clinical studies.

Prior Transient Ischemic Attack or Stroke: In total, 262 subjects of 6484 in the prasugrel group and 256 subjects of 6464 in the clopidogrel group had a prior history of TIA or stroke. Subjects with a history of prior TIA or stroke and treated with prasugrel had a statistically significant higher incidence of nonfatal stroke (15/262 (5.73%) vs. 2/256 (0.78%), $p < 0.001$) and all stroke (both, fatal and nonfatal, hemorrhagic or non-hemorrhagic) (17/262 (6.49%) vs. 3/256 (1.17%), $p < 0.001$), when compared to clopidogrel. A similar, statistically significant pattern was observed in the UA/NSTEMI population, whereas the number of stroke events in the STEMI population with prior history of TIA/stroke was too low to allow any reliable conclusions. In addition, a history of prior TIA/stroke in the all ACS population was associated with a higher risk of Non-CABG-related TIMI Major or Minor bleeding events (Prasugrel: 20/257 (7.78%) vs. clopidogrel: 10/252 (3.97%), $p = 0.054$) and of Non-CABG-related TIMI Major Life-Threatening bleeding events (11/257 (4.28%) vs. 3/252 (1.19%), $p = 0.026$, including fatal bleeding and symptomatic ICH) with prasugrel therapy. Regarding the fatal ICH, 2 out of 9 patients in the prasugrel group had a prior history of TIA/stroke vs 0 out of 5 patients in the clopidogrel group. The higher bleeding risk in patients with prior TIA or stroke was not associated with higher exposure during MD. Thus, patients with a prior history of TIA or stroke are contraindicated for prasugrel.

Low body weight: The risk of Non-CABG-related TIMI Major or Minor bleeding events for patients weighing below 60 kg was greater for prasugrel treated patients compared to clopidogrel treated, though not significantly different. However, the number of patients weighing less than 60 kg was very low (664 subjects in total in both treatment groups). PK data has shown that the active metabolite exposure increases as body weight decreases (see section on clinical pharmacology). A lower prasugrel 5 mg MD for this subgroup could be used. A PK/PD study to investigate the 5 mg dose in this patient population will be conducted as part of the FUMs.

Ethnicity: Prasugrel active metabolite exposure in Asian subjects was 43% higher after a 60-mg prasugrel LD and 40% higher during 10-mg prasugrel MD compared to Caucasian subjects. Based on point estimates for comparisons between Asians and Caucasians, body weight accounted for about one-third of the exposure difference between the two ethnic groups. There were so few Non-CABG-related Major or Minor bleeding events in the non-Caucasian populations that a meaningful comparison could not be done. A new PK/PG study in approximately 715 Asian ACS subjects in order to clarify which could be the optimal dose for this population will be conducted. Until this data become available a cautious approach is appropriate by including a warning in the SPC.

- Safety related to drug-drug interactions and other interactions
- Specific *in vivo* drug-interaction studies were conducted with prasugrel and aspirin, ketoconazole (a potent CYP3A inhibitor), rifampicin (a potent inducer of CYP3A and CYP2B6 and an inducer of CYPs 2C9, 2C19, and 2C8), atorvastatin (a statin metabolized by CYP3A), warfarin (an anti-coagulant metabolized by CYPs 2C9 and 2C19), heparin, bupropion (a CYP2B6 substrate), and digoxin (a P-glycoprotein [P-gp] substrate). The effect of smoking and alcohol consumption were also evaluated across clinical pharmacology studies. Overall, these analyses detected no clinically relevant drug interactions.
- Proton pump inhibitors may slow the rate, but not the extent, of appearance of prasugrel's active metabolite in plasma. Prasugrel can be co-administered with a proton pump inhibitor (PPI) or a H₂-receptor antagonist. However, the SPC was revised to state that administration of the loading dose without co-administration with PPI may provide most rapid onset of action.

- Discontinuation due to adverse events

In the All ACS populations, overall incidence of study drug discontinuation due to treatment-emergent adverse events (TEAEs) was higher in subjects treated with prasugrel (462/6741 (7.15%)) compared to clopidogrel (390/6716 (6.02%)). The rate of discontinuation of study drug due to an AE was similar between treatment groups through 90 days, at which time the Kaplan-Meier curves diverge in favour of clopidogrel. The higher incidence of study drug discontinuation with prasugrel due to AE was primarily due to the higher incidence of hemorrhagic events (with GI hemorrhage (33/3741 (0.49%) vs. 21/6716 (0.32%)) and epistaxis (0.31% vs. 0.12%) being the most common). Atrial fibrillation (20/6741 (0.31%) vs. 33/6716 (0.51)) and rash (0.28% vs. 0.42%) were the non-hemorrhagic events leading to the highest incidence of permanent study drug discontinuation, but the rate between the 2 treatment groups was similar. In the secondary database there were no observed treatment differences between prasugrel and clopidogrel in the incidence of SAEs and non-serious TEAEs leading to premature discontinuation of study drug.

- Post marketing experience

There is currently no post-marketing experience with the use of this product.

- Discussion on clinical safety

The key safety findings associated with prasugrel treatment were a statistically higher incidence of hemorrhagic AEs. Adjudicated non-CABG-related TIMI Major Bleeding, TIMI Major or Minor Bleeding and TIMI Major, Minor, or Minimal Bleeding were statistically significantly increased in the prasugrel group compared to the clopidogrel group as well as fatal hemorrhagic AEs which were also higher in the prasugrel group. The Kaplan-Meier curves separated early for Non-CABG-related TIMI Major and TIMI Life-Threatening bleeding events while at risk, favouring clopidogrel. It seems that the curves remained parallel between 90 and 360 days. However, between 30 and 90 days as well as beyond 360 days, events continued to increase in subjects treated with prasugrel while a diminished accrual rate was seen in subjects treated with clopidogrel. Patients for whom a 10 mg maintenance dose is not recommended (those with a history of TIA or stroke – contraindication-, subjects ≥ 75 years of age taking a MD of 10 mg/day, or subjects < 60 kg taking a MD of 10 mg/day) are responsible for the accrual between 30 and 90 days. The apparent accrual beyond 350 days cannot be explained. At the same time, the clinical benefit of prasugrel beyond 12 months seems not sufficiently supported by clinical data, this is why the CHMP has recommended that dual antiplatelet therapy with prasugrel should be restricted to 12 months treatment duration, in line with current clinical recommendations for dual antiplatelet therapy. This was also accepted by the Scientific Advisory Group of the CHMP.

The observation that fatal bleedings were higher in the prasugrel group compared to the clopidogrel group was of concern. However, the number of the spontaneous fatal bleeding events was similar between treatment groups, especially considering the ideal population for whom the prasugrel 10-mg MD would be recommended (subjects without a history of TIA or stroke, subjects ≥ 75 years of age, or subjects < 60 kg). The rate of Non-CABG-related TIMI Major or Minor bleeding events in the ideal population was not statistically different between treatment groups but numerically higher for prasugrel. Though numbers of patients undergoing CABG were small, the risk of CABG related TIMI Major or Minor Bleeding was approximately tripled in the prasugrel arm, in particular in patients undergoing CABG within 7 days of the last dose of study drug. Bleeding events were also the primary reason for treatment discontinuations.

Subgroups associated with a statistically significant increase in Non-CABG-related TIMI Major bleeding were: ≥ 75 years old, body weight < 60 kg, and history of prior TIA or stroke. The lastly mentioned patients have been contraindicated. For patients < 60 kg the events were associated with higher exposure of the active metabolite, supporting the proposed prasugrel 5-mg MD in this subgroup. For patients ≥ 75 years the events were partly associated with increased exposure to the active metabolite along with a greater susceptibility to bleeding. A dose adjustment strategy was justified by further analyses and planned future clinical studies for both the low weight patients and the very elderly. The use of prasugrel in patients ≥ 75 years of age is generally not recommended. If, after a careful individual benefit/risk evaluation by the prescribing physician, treatment is deemed necessary in the ≥ 75 years age subgroup then following a 60 mg loading dose, a reduced prasugrel 5 mg MD should be prescribed.

The evidence for the 5 mg MD is based only on PK/PD analyses and no clinical data currently exist on the safety of this dose in the ≥ 75 years age group.

2.5 Pharmacovigilance

Detailed description of the Pharmacovigilance system

The CHMP considered that the Pharmacovigilance system as described by the applicant fulfils the legislative requirements.

Risk Management Plan

The MAA submitted a risk management plan, which included a risk minimisation plan.

Table Summary of the risk management plan

Safety concern	Proposed pharmacovigilance activities	Proposed risk minimisation activities
Identified Risks		
1. Haemorrhage: (Intracranial haemorrhage, Gastrointestinal haemorrhage, Intraocular haemorrhage, Percutaneous Coronary Intervention-Related Haemorrhage, Coronary Artery Bypass Graft-Related Haemorrhage, Other Procedure-Related Haemorrhage, Epistaxis)	<ul style="list-style-type: none"> • Routine pharmacovigilance: monitor AEs and SAEs through routine clinical trial and spontaneous post-marketing surveillance. • Targeted surveillance for specific AEs preidentified for targeted follow-up. • In-hospital registry to monitor prasugrel use and bleeding risk during the index hospitalisation compared to clopidogrel in a real life EU clinical setting. 	<ul style="list-style-type: none"> • Contraindication for patients with history of stroke or transient ischaemic attack (TIA) and for patients with active pathological bleeding in Section 4.3 of SPC. • Section 4.2: dose adjustment for patients with risk factors for increased risk of bleeding: patients ≥ 75 years of age and patients <60 kg. • Wording in sections 4.2 and 4.4 of the SPC regarding the restricted use of EFIENT in patients ≥ 75 years of age, and maintenance dose reduction in these patients. • Caution for patients with a propensity to bleed, and with risk factors for an increased risk of bleeding (section 4.4) • Caution with concomitant administration of medicinal products that may increase the risk of bleeding (section 4.4) • Further recommendations to minimise the risk of haemorrhage, including CABG-related haemorrhage, are given in Section 4.4 (Surgery) and Section 4.8 of the SPC. Section 4.4 of the SPC recommends discontinuation of EFIENT at least 7 days prior to surgery. • Epistaxis is listed as ADR in Table 2, Section 4.8 of SPC. • Additional risk minimisation for patients ≥ 75 years of age will be provided by health care professional education to ensure that the information and recommendations in the SPC are adequately communicated. The MAH commits to work with scientific societies to develop educational vehicles for this purpose.
2. Anaemia	<ul style="list-style-type: none"> • Routine pharmacovigilance: monitor AEs and SAEs through routine clinical trial and spontaneous post-marketing surveillance. • Targeted surveillance for specific AEs preidentified for targeted follow-up. 	<ul style="list-style-type: none"> • Listed as ADR in Table 2, Section 4.8 of SPC.

Safety concern	Proposed pharmacovigilance activities	Proposed risk minimisation activities
Potential Risks		
Risks associated with off-label use	<ul style="list-style-type: none"> • Routine pharmacovigilance: monitor AEs and SAEs through routine clinical trial and spontaneous post-marketing surveillance. • Targeted surveillance for specific AEs preidentified for targeted follow-up. • In –hospital registry to monitor prasugrel use and bleeding risk during the index hospitalisation compared to clopidogrel in a real life EU clinical setting. • Off-Label Use in Patients Post-Discharge: To monitor the off-label use post-discharge in patients treated with prasugrel. The databases will capture data pertaining to drug utilisation to monitor in what patients prasugrel is used, and at what doses. 	<ul style="list-style-type: none"> • Not in proposed SPC as not confirmed signal.
Phototoxicity (Skin or Ocular)	As bullet 1 above.	<ul style="list-style-type: none"> • Not in proposed SPC as not confirmed signal.
Drug-Induced Hepatic Injury	As bullets 1 and 2 above.	<ul style="list-style-type: none"> • Not in proposed SPC as not confirmed signal.
Allergic Reactions	As bullets 1 and 2 above.	<ul style="list-style-type: none"> • Not in proposed SPC as not confirmed signal.
Thrombocytopenia	As bullets 1 and 2 above.	<ul style="list-style-type: none"> • Not in proposed SPC as not confirmed signal.
Neutropenia	As bullets 1 and 2 above.	<ul style="list-style-type: none"> • Not in proposed SPC as not confirmed signal.
Thrombotic Thrombocytopenic Purpura	As bullets 1 and 2 above.	<ul style="list-style-type: none"> • Warning in Section 4.4 of the SPC includes a description of this serious event.

Safety concern	Proposed pharmacovigilance activities	Proposed risk minimisation activities
Missing Information		
Concomitant use with fibrinolytics, clopidogrel, and chronic use of NSAIDs (non ASA).	<ul style="list-style-type: none"> Continue to analyse AE reports in clinical trials Periodically review and analyse safety database Any spontaneously reported case associated with an exposure condition (EC) is managed according to internal procedures for clarification. Safety surveillance intends to identify signals associated with the ACS subpopulations and use of drugs associated with an increased risk of bleeding. Concomitant drug use will also be monitored in the in-hospital registry. 	<ul style="list-style-type: none"> Sections 4.4 and 4.5 of the SPC contain language cautioning against concomitant use with these drugs.
Paediatric population	<ul style="list-style-type: none"> Continue to analyse paediatric data from clinical trials Periodically review and analyse safety database for any potential post-marketing use in the paediatric or adolescent population. 	<ul style="list-style-type: none"> Section 4.2 of SPC states that EFIENT is not recommended for use in children and adolescents due to a lack of data on safety and efficacy.
Pregnant/Lactating women	<ul style="list-style-type: none"> Continue to analyse AE reports in clinical trials Periodically review and analyse safety database for any potential post-marketing use in pregnant or lactating women Routine pharmacovigilance, targeted surveillance with specific follow-up form for pregnancy and lactation, safety surveillance for safety signal detection associated with these events. Pharmacovigilance, targeted surveillance with specific follow-up form for exposure condition, safety surveillance for safety signal detection associated with the exposure condition. 	<ul style="list-style-type: none"> Section 4.6 of SPC recommends a risk/benefit evaluation approach with regard to pregnancy, and does not recommend use of EFIENT during breastfeeding.

Summary of the Risk Management Plan for EFIENT (prasugrel) – concluded

Safety concern	Proposed pharmacovigilance activities	Proposed risk minimisation activities
Missing Information (cont.)		
Subjects without clinical manifestation of ACS or with ACS not managed by PCI (requiring immediate CABG or suitable for medical management only)	<ul style="list-style-type: none"> Continue to analyse AE reports in clinical trials Periodically review and analyse safety database for any spontaneously reported case associated with these situations CAD subjects with no symptom of ACS may be detected by routine pharmacovigilance activities. Medically-managed ACS subjects not planned to be managed by PCI will be studied in Study TABY. 	<ul style="list-style-type: none"> Indication statement in Section 4.1 of the SPC includes definition of the targeted population for EFIENT, i.e. ACS <i>undergoing</i> PCI.
Subjects with severely compromised cardiac status (cardiogenic shock, Class IV CHF, refractory ventricular arrhythmia)	<ul style="list-style-type: none"> Routine pharmacovigilance, safety surveillance for safety signal detection associated to prasugrel use in this specific subpopulation. 	<ul style="list-style-type: none"> Indication statement in Section 4.1 of the SPC describes the target patient population as follows: <ul style="list-style-type: none"> patients with acute coronary syndrome (i.e. unstable angina, non-ST segment elevation myocardial infarction [UA/NSTEMI] or ST segment elevation myocardial infarction [STEMI]) undergoing primary or delayed percutaneous coronary intervention (PCI).
Subjects with severe hepatic impairment.	<ul style="list-style-type: none"> Continue to analyse AE reports in clinical trials Periodically review and analyse safety database for any spontaneously reported case associated with severe hepatic impairment In –hospital registry will allow for identification of subjects with possible liver damage. 	<ul style="list-style-type: none"> Contraindication for patients with “severe hepatic impairment (Child Pugh Class C)” in Section 4.3 of the SPC.

The CHMP, having considered the data submitted in the MA application is of the opinion that the following risk minimisation activities are necessary for the safe and effective use of the medicinal product:

The MAH should provide educational material to all physicians who may be involved in treating patients with prasugrel. The format and means of dissemination, of this material should be discussed with the appropriate learned societies. The results of the discussion, and where appropriate the material, should be agreed with the national competent authority and be available prior to launch in each member state.

The educational material should include:

- A copy of the SPC
- Emphasis that:

- Severe haemorrhagic events are more frequent in patients ≥ 75 years of age (including fatal events) or those weighing < 60 kg
- Treatment with prasugrel is generally not recommended for patients of ≥ 75 years of age.
- If, after a careful individual benefit/risk evaluation by the prescribing physician, treatment is deemed necessary in the ≥ 75 years age group then following a loading dose of 60 mg, a reduced maintenance dose of 5mg should be prescribed.
- Patients weighing < 60 kg should have a reduced maintenance dose of 5mg
The evidence for a 5mg dose is based only on PK/PD analyses and no clinical data currently exist on the safety of this dose in the at risk sub groups.

2.6 Overall conclusions, risk/benefit assessment and recommendation

Quality

The quality of the product is considered to be acceptable when used in accordance with the conditions defined in the SPC. Physicochemical and biological aspects relevant to the uniform clinical performance of the product have been investigated and are controlled in a satisfactory way. There are a number of quality issues that will be resolved as Follow-up Measures within an agreed timeframe. None of these issues is expected to have a negative impact on the Benefit Risk balance of the product.

Non-clinical pharmacology and toxicology

The non clinical pharmacological programme provided an adequate characterisation of the pharmacological properties of prasugrel. In *ex vivo* studies with rats, dogs, and cynomolgus monkeys, prasugrel demonstrated dose-dependent inhibition of ADP-induced platelet aggregation. The *in vivo* effects of prasugrel were assessed in nonclinical pathophysiological models of thrombotic challenge. Administration of prasugrel at clinical doses is not expected to produce secondary pharmacology effects related to CNS, cardiovascular, respiratory, renal, or GI function or to have an affect on QT interval. Additive or synergistic platelet inhibitory effects of the co-administration of prasugrel with aspirin have also been demonstrated. The active metabolite of prasugrel R-138727 is chiral; with two most potent enantiomers of R-138727 comprise approximately 84% of the metabolite in human plasma. The overall metabolism of prasugrel was thoroughly investigated.

The primary effects of prasugrel observed during repeat-dose toxicology studies included decreased body weight relative to control in rodents that was occasionally accompanied by decreased food consumption, and increased liver weight and histologic changes in the liver considered to be related to microsomal enzyme induction. Prasugrel did not exhibit genotoxic properties when tested *in vitro* nor *in vivo*. The increase in liver tumours observed in mice dosed with prasugrel is not considered a relevant human risk, and this is adequately reflected in the proposed prescribing information. Prasugrel did not exhibit toxicity towards fertility and early embryonic development and did not show embryo-foetal toxicity. The SPC obtains adequate statements.

Non-clinical and clinical data indicate that evidence of the phototoxic potential of prasugrel is weak and of questionable clinical relevance. Nevertheless, phototoxicity was included as a potential risk in the RMP.

A complete environmental risk assessment has been conducted for prasugrel. No likely risk has been identified for aquatic organisms in either ground water or surface water, or for sediment dwelling organisms.

Efficacy

A single pivotal superiority trial supports the use of prasugrel (60 mg LD and 10 mg MD) in patients with ACS with scheduled PCI. This study demonstrated that treatment with prasugrel, as compared with clopidogrel at the standard approved dose resulted in a statistically significant reduction in the rate of the primary composite efficacy endpoint of cardiovascular death, nonfatal MI, or nonfatal stroke. The reduction of the incidence of primary composite endpoint was primarily driven by a

reduction in the number of cardiac ischemic events, in particular nonfatal MI. These events are not considered to be harmless periprocedural increases in biochemical markers after index PCI alone. Prasugrel treatment might initially protect against smaller myocardial infarctions related to index PCI, however, the absolute reduction of nonfatal myocardial infarction in the all ACS population continued to increase throughout the study period. The absolute percentage reductions were 0.94, 1.57, 1.65, and 2.15 at 3 days, 30 days, 90 days, and at study end, respectively. When similar analyses are done separately in UA/NSTEMI and STEMI populations, continued increases in the absolute reduction of nonfatal myocardial infarction were also observed. This data suggests that prasugrel treatment protects against short-term as well as long-term cardiac ischemic events. A relative risk reduction of about 20% was observed in UA/NSTEMI, STEMI, and all ACS populations. The approximate 2% absolute risk reduction observed in these populations is considered clinically meaningful.

Data from subgroup analyses suggest that patients with a history of diabetes mellitus could benefit from prasugrel treatment. In response to a question on this topic from the CHMP, a general discussion of platelet reactivity and ischemic risk in diabetic patients in the setting of ACS preceded a presentation of clinical outcomes in subjects with diabetes mellitus in study TAAL. Diabetic subjects, not on insulin, had higher relative and absolute risk reduction if randomized to prasugrel, compared to non-diabetic patients randomized to prasugrel. Even higher risk reductions were observed in diabetic patients on insulin, with the absolute risk reduction (primary efficacy endpoint) with prasugrel vs clopidogrel being 6.4%. The relative risk reduction was 37% in this sub-population. Qualitatively similar effects of diabetic status were observed for a number of other efficacy endpoints. Diabetic status seems to affect efficacy and safety differentially, favouring prasugrel treatment in diabetic subjects in particular. It is also acknowledged that consistency of the efficacy benefit across pre-specified primary and secondary endpoints supports the notion that the treatment benefit in diabetic subjects is not a chance finding. However, in contrast the clinical data indicate that patients with a history of prior TIA or stroke are harmed by treatment with prasugrel when compared to treatment with clopidogrel. This effect on the primary efficacy endpoint seems to be driven primarily by an increase in new strokes. For this reason, a history of prior stroke or TIA is now listed under contraindications in the SPC.

In the < 60 kg group, a reduced maintenance dose of 5 mg following a 60 mg loading dose should be prescribed. If treatment is deemed necessary in the ≥ 75 years age group, a reduced maintenance dose of 5 mg following a 60 mg loading dose should be prescribed after a careful individual benefit/risk evaluation by the prescribing physician. There are no adequate clinical data to support this recommendation; thus, the positive clinical outcome of this reduced maintenance dose remains to be seen. Further clinical studies to address this issue will be conducted.

Safety

The safety of prasugrel was comparable to clopidogrel with respect to the incidence of AEs, SAEs and deaths, as well as pre-specified, non-hemorrhagic, clinically relevant TEAEs and laboratory values (thrombocytopenia, neutropenia, leucopenia, allergic reactions, including angioedema, abnormal hepatic function, and torsades de pointes/QT prolongation). The overall incidence of study drug discontinuation was higher in the prasugrel group compared to clopidogrel (approximately 1% greater) primarily due to a higher incidence of hemorrhagic AEs.

Adverse events related to haemorrhage occurred with a statistically significantly higher incidence in the prasugrel treated patients compared to clopidogrel and this was consistent in the majority of subgroups of bleeding events.

Recent clinical studies of anti-thrombotic agents have suggested that major bleeding events may predict an increased risk of CV or non-CV death in the early weeks following the bleeding event and the administration of blood units are called to play a special role on this. This increased risk is also evident in study TAAL. Additionally in this regard, there is ongoing controversy about the efficacy and safety of blood transfusions in the ACS context. To the extent that current recommendations indicate that in mild to moderate anaemia (Hct $>25\%$ and Hb >8 g/dl) blood transfusions may be related with an increase risk of death at 30 days and should be avoided if haemodynamically well tolerated. As mentioned earlier, in a post hoc analysis in Study TAAL assessment of long-term

outcomes for subjects experiencing a Non-CABG-related TIMI Major bleeding event indicated that, after 30 days from the event, the risk for major adverse CV events was not higher than that observed in subjects not experiencing the bleeding event. Beyond 30 days, mortality was comparable in those patients who did not have a major bleeding and those who had a major bleeding. Likewise, within 30 days, mortality was comparable for prasugrel and clopidogrel treated patients who did not experience a major bleeding. However, after a TIMI major bleeding, mortality (within 30 days) was significantly higher for prasugrel treated patients compared to clopidogrel treated patients for the All ACS population and its clinical presentations UA/NSTEMI and STEMI. When considering only the so-called ideal population (patients without a history of TIA or stroke, subjects ≥ 75 years of age, or subjects < 60 kg), all cause mortality is still numerically higher after TIMI major bleeding in prasugrel compared to clopidogrel but the difference was not statistically significant. The deaths were predominantly attributable to fatal bleedings.

Apart from the direct haemodynamic consequences of the bleeding episode, which is also associated with the risk of ischemic events occurring in relation to bleeding induced hypotension or transfusions, an important component of the risk is the potential need to interrupt the antiplatelet and antithrombotic drugs which can lead to an increase of ischemic events. In clinical practice the risk of interrupting antithrombotic and antiplatelet treatments must be weighed against the risk of a thrombotic event, particularly if the patient has been submitted to revascularization and stent implantation. The SPC states that premature discontinuation of any antiplatelet agent could result in increased risk of ischaemic events. Further, the SPC includes a warning that in patients with active bleeding in whom reversal of pharmacological effects of prasugrel is required, platelet transfusion may be appropriate.

Colon cancer occurred with a higher incidence in patients treated with prasugrel, but this was assumed to be the consequence of a higher rate of detection rate due to bleeding associated with this therapy.

From the safety database all the adverse reactions reported in clinical trials have been included in the Summary of Product Characteristics.

Having considered the safety concerns in the risk management plan, the CHMP considered that the proposed activities described in section 2.5 adequately addressed these concerns

- **User consultation**

User testing of the package leaflet was performed using methodology, which methodology follows the readability guideline. No revisions of the PL were made between test rounds. In conclusion, the user testing of this PL version is judged acceptable.

Risk-benefit assessment

Superiority of prasugrel over clopidogrel was shown in the clinical setting when the primary composite efficacy endpoint - CV death, nonfatal MI, or nonfatal stroke at a median follow-up of 14.5 months - is considered, and was primarily driven by a reduction in MIs. Peri-procedural as well as spontaneous MIs were decreased with prasugrel.

The treatment benefit associated with prasugrel was generally preserved across the major pre-specified subgroups. The reduction in ischemic events with prasugrel was evident regardless of the adjunctive therapy or stent type selected during PCI. Clinical data suggest that patients with a history of diabetes could benefit in particular from treatment with prasugrel. Vice versa, although not reaching statistical significance, the data also suggest that subjects with a history of prior TIA or stroke are harmed by treatment with prasugrel compared to treatment with clopidogrel. This patient subgroup is contraindicated.

Key safety issues were primarily related to the risk of bleeding. A statistically higher incidence of hemorrhagic AEs was observed for prasugrel vs clopidogrel. These were related to higher rates of GI bleeding, surgical site bleeding, bleeding at other sites not pre-specified or unknown and retroperitoneal bleeding. Fatal hemorrhagic AEs were higher in the prasugrel group. The finding of significantly increased hemorrhagic events in the prasugrel studies was in general seen for the All-

ACS population and the UA/NSTEMI population. In the STEMI subgroup incidences of the different hemorrhagic events were in general numerically higher in the prasugrel group compared to the clopidogrel group, however, the differences were in most cases not statistically significant. Subgroups vulnerable to bleeding were patients ≥ 75 years old, patients with body weight < 60 kg, and patients with a history of prior TIA or stroke. Further analyses indicated that increased mortality after TIMI major bleedings with prasugrel within the first month of treatment appeared to be related mainly to fatal bleedings. The higher bleeding risk associated with the use of prasugrel is appropriately described in the SPC and guidance has been given to minimise the use of prasugrel in populations at higher risk of bleeding.

It should also be remembered that safety in a real clinical setting tends to be worse than observed under controlled conditions. Attention should therefore be paid to the possible risk of ischemic events which may occur in relation to major bleeding events, e.g. by discontinuation of antiplatelet therapy, bleeding induced hypotension, or transfusions.

For patients ≥ 75 years, treatment is generally not recommended. For selected subgroup of patients ≥ 75 years for whom prasugrel treatment is deemed necessary, a reduced maintenance dose of 5 mg should be prescribed. Patients < 60 kg should receive a reduced maintenance dose of 5 mg. The main limitation of this recommendation for a prasugrel dose adjustment is the current lack of clinical data supporting the safety and efficacy for the treatment of these subgroups with a reduced maintenance dose of 5 mg. Additional clinical studies to assess the 5 mg dose in the relevant at risk populations are necessary and are planned, as discussed earlier.

In summary, the following table describes Number Needed to Treat (NNT) and Number Needed to Harm (NNH) for different subgroups.

NNT (Primary Efficacy Outcome) and NNH (TIMI Major bleedings) - Study TAAL; All randomized Subjects

Subgroup	Efficacy Sample size	NNT (Primary efficacy¹)	NNT (All death, MI, Stroke)	NNH (TIMI Major)	NNH (TIMI Life-Threatening)
All ACS	13608	49	52	195	234
UA/NSTEMI	10074	52	58	163	186
STEMI	3534	42	40	444	888
Gender					
Female	3523	72	59	109	226
Male	10085	44	50	254	229
Age					
≥75 years	1809	102	192	110	98
<75 years	11799	45	47	220	295
Weight					
<60 kg	668	81	58	36	81
≥60 kg	12769	48	52	267	284
Region					
North America	4310	35	36	328	1243
Western EU	3553	73	70	198	222
Eastern EU	3322	61	75	102	163
Medical History					
Diabetics	3146	23	22	795	313
Non-diabetics	10462	74	87	159	218
Prior TIA/Stroke	518	-23	-20	33	32
No prior TIA/Stroke	13090	43	45	243	311
Ideal population²	10,804	39	40	312	368

¹ CV death, non-fatal MI, or non-fatal stroke

² that is, population for whom a 10 mg MD is recommended

Sufficient evidence has been provided that the timing of clopidogrel LD, which is recommended to be given immediately, and not, as in study TAAL after diagnostic angiography, did not substantially influence the efficacy of prasugrel vs clopidogrel.

A risk management plan was submitted. The CHMP, having considered the data submitted, was of the opinion that:

- pharmacovigilance activities in addition to the use of routine pharmacovigilance were needed to investigate further some of the safety concerns.

- the following additional risk minimisation activities were required:

The MAH should provide educational material to all physicians who may be involved in treating patients with prasugrel. The format and means of dissemination, of this material should be discussed with the appropriate learned societies. The results of the discussion, and where appropriate the material, should be agreed with the national competent authority and be available prior to launch in each member state.

The educational material should include:

- A copy of the SPC
- Emphasis that:
 - Severe haemorrhagic events are more frequent in patients ≥ 75 years of age (including fatal events) or those weighing < 60 kg
 - Treatment with prasugrel is generally not recommended for patients of ≥ 75 years of age.

- If, after a careful individual benefit/risk evaluation by the prescribing physician, treatment is deemed necessary in the ≥ 75 years age group then following a loading dose of 60 mg, a reduced maintenance dose of 5mg should be prescribed.
- Patients weighing < 60 kg should have a reduced maintenance dose of 5mg
The evidence for a 5mg dose is based only on PK/PD analyses and no clinical data currently exist on the safety of this dose in the at risk sub groups.

Recommendation

Based on the CHMP review of data on quality, safety and efficacy, the CHMP considered by consensus decision that the risk-benefit balance of Effient “co-administered with acetylsalicylic acid (ASA), for the prevention of atherothrombotic events in patients with acute coronary syndrome (i.e. unstable angina, non-ST segment elevation myocardial infarction [UA/NSTEMI] or ST segment elevation myocardial infarction [STEMI]) undergoing primary or delayed percutaneous coronary intervention (PCI)” was favourable and therefore recommended the granting of the marketing authorisation.

Exhibit 37

Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Prasugrel

Stephen D. Wiviott, Elliott M. Antman and Eugene Braunwald

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Prasugrel

Stephen D. Wiviott, MD; Elliott M. Antman, MD; Eugene Braunwald, MD

Platelet adhesion, activation, and aggregation play key roles in both normal hemostasis and pathological thrombosis. In the latter, these factors are paramount in the initiation of the intracoronary thromboses that cause acute coronary syndromes (ACS) and the ischemic complications following coronary artery interventions, including recurrent myocardial infarction (MI) and stent thrombosis.¹ The interaction of ADP with purenergic P2Y₁ and P2Y₁₂ receptors serves to amplify and sustain platelet activation.² Activated platelets expose glycoprotein IIb/IIIa receptors, which crosslink with fibrin to form platelet aggregates. These aggregates cause mechanical disruption of flow and may embolize downstream, causing microvasculature obstruction that results in myocardial ischemia and infarction.

The important role of antiplatelet agents in the management and prevention of the complications after ACS and percutaneous coronary intervention (PCI) is related directly to the physiological events noted above.^{3–7} Thienopyridine antiplatelet agents interfere with platelet activation and aggregation induced by ADP. There are 3 members of the thienopyridine class of antiplatelet agents currently available for clinical use: ticlopidine, clopidogrel, and the subject of this review, prasugrel. All 3 agents are prodrugs and require conversion to an active metabolite to exhibit an antiplatelet effect (Figure 1). The active metabolite of the thienopyridine binds irreversibly to the P2Y₁₂ receptor, blocking the binding of ADP and thereby inhibiting platelet activation and aggregation⁸ and leading to the clinical benefits and risks of these agents. The benefits of ticlopidine were shown in a series of trials comparing dual antiplatelet therapy with aspirin plus an oral anticoagulant.^{9,10} Ticlopidine is limited by the need to take the drug twice daily, by poor tolerability, notably gastrointestinal distress, but most important by severe side effects, including bone marrow aplasia.¹¹ Clopidogrel plus aspirin dual antiplatelet therapy has become the standard of care for the support of patients undergoing PCI with stenting largely on the basis of a better tolerability profile.^{12,13} Clinical trials established the benefits of clopidogrel across the spectrum of ACS, including unstable angina (UA), non-ST-elevation MI (NSTEMI), and ST-elevation MI (STEMI).^{14–16} American College of Cardiology/American Heart Association guidelines recommend dual antiplatelet therapy with aspirin and clopidogrel in patients with ACS for up to 1 year

regardless of syndrome type or treatment strategy (medical, PCI, or surgery).⁶

Pharmacological Limitations of Clopidogrel

Despite the major benefits of clopidogrel alone and in combination with aspirin for patients with ACS and for those undergoing PCI, important pharmacological limitations are associated with its use.¹⁷ The antiplatelet effects of clopidogrel have a delayed onset (several hours after ingestion), and there is substantial variability in response among patients. A growing number of studies have linked poor antiplatelet response to clopidogrel to adverse clinical outcomes, particularly coronary ischemia and stent thrombosis.^{18–21} From these limitations, the evaluation of more intensive and consistent antiplatelet therapy compared with clopidogrel has been fostered. One such agent, prasugrel, a third-generation thienopyridine, is the focus of this review.

Pharmacology of Prasugrel

Prasugrel requires enzymatic metabolism to exert its antiplatelet effects (Figure 1).²² The parent molecule, prasugrel, is rapidly hydrolyzed by esterases, such as those located in the intestine and blood, to a thiolactone intermediate metabolite R-95913. The parent molecule is therefore not detectable in the plasma. This intermediate metabolite undergoes subsequent activation by a single cytochrome P450 (CYP)-dependent step (predominantly CYP3A and CYP2B6) to form the sulfhydryl-containing active metabolite R-138727. This active metabolite binds irreversibly to the platelet P2Y₁₂ receptor by covalent linkage of a sulfhydryl group to inhibit platelet activation and aggregation. Prasugrel metabolism differs from clopidogrel metabolism in that the initial hydrolysis of the parent clopidogrel compound results in inactivation of a substantial fraction (≈85%) of the absorbed drug, and the subsequent activation requires 2 CYP-dependent steps (Figure 1).²² The CYP enzymes involved in the metabolism of clopidogrel and prasugrel are polymorphic, differing between individuals, and are responsible in part for the interpatient variability of clopidogrel response.^{23,24} The prasugrel active metabolite concentration peaks in the plasma at ≈30 minutes, and the concentration is proportional to a dose between 5 and 60 mg. When not bound to platelets, the active metabolite of prasugrel has an elimination half-life of ≈7 hours.²⁵ Prasugrel does

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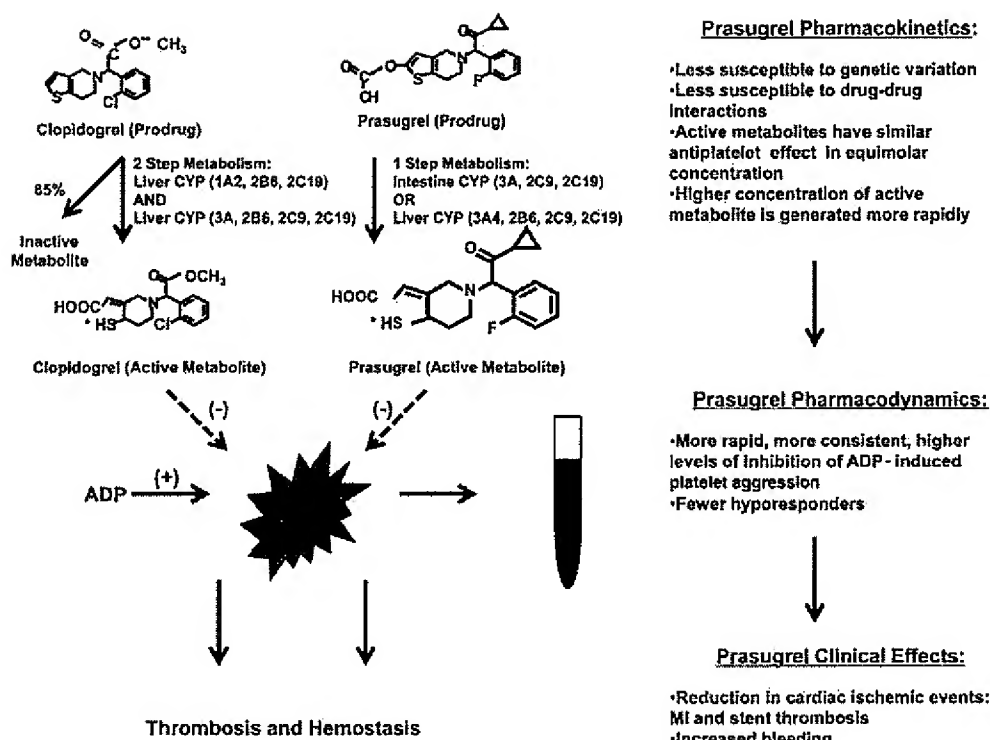


Figure 1. Schematic representation of the relationship between thienopyridine metabolism, pharmacological effects, and clinical outcomes.

not have clinically relevant interactions with inducers or inhibitors of the cytochrome P450 system.²⁶ The active metabolite concentration and pharmacodynamic response of prasugrel were not affected by moderate renal impairment compared with healthy subjects.²⁷ In patients with end-stage renal disease, active metabolite concentrations were $\approx 40\%$ lower, but similar platelet inhibition was noted. In patients with moderate liver disease, no effects on prasugrel pharmacokinetics or pharmacodynamics have been observed. Prasugrel has not been tested in severe hepatic disease.²⁸

In clinical pharmacology studies of healthy subjects, no effect of age on prasugrel pharmacokinetics or pharmacodynamics was observed with age between 20 and 80 years.²⁹ In the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38), however, patients ≥ 75 years had 19% higher exposure to the active metabolite of prasugrel compared with those < 75 years of age and 25% higher exposure compared with patients < 60 years of age. In both clinical pharmacology studies and TRITON-TIMI 38, prasugrel pharmacokinetics were affected by body weight, with higher exposure in subjects with lower body weight. In TRITON-TIMI 38, patients < 60 kg had 30% higher exposure than patients ≥ 60 kg and 42% higher exposure than patients ≥ 85 kg. In the analysis of TRITON-TIMI 38, pharmacokinetics were not appreciably affected by diabetes, smoking, or renal impairment.³⁰

In equimolar concentrations, the active metabolites of prasugrel and clopidogrel have similar antiplatelet effects.³¹ Therefore, the more rapid and consistent conversion of prasugrel from the inactive prodrug to its active metabolite and the ability of patients to generate higher concentrations of

the active metabolite provide the mechanistic basis for the differences in the pharmacological profiles of the 2 drugs.³² A 2-phase crossover study comparing loading doses of prasugrel (60 mg) and clopidogrel (300 mg) in healthy subjects showed higher maximal levels and less variable inhibition of ADP-induced platelet aggregation with prasugrel ($79 \pm 9\%$) than with clopidogrel ($35 \pm 25\%$).³² A difference in platelet inhibition was apparent between the 2 drugs by 30 minutes and persisted to 24 hours, the duration of measurement. This same study also demonstrated several patients with low-level ($< 20\%$ induced platelet aggregation) response to clopidogrel but no such patients with prasugrel. These observations resulted from the ≈ 10 -fold higher levels of prasugrel active metabolite than clopidogrel active metabolite.³²

Many physicians in clinical practice use higher doses of clopidogrel than the standard approved dose. This practice is supported in part by clinical practice guidelines,¹² small clinical outcomes studies, observational studies, and meta-analyses.^{33–35} The Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent Events/Optimal Antiplatelet Strategy for Interventions (CURRENT OASIS 7) trial³⁶ compared high-loading- and -maintenance-dose clopidogrel (600-mg loading dose followed by 150 mg/d for 7 days) with standard dosing (300-mg loading dose followed by 75 mg/d) in ≈ 25 000 patients with ACS treated with or without coronary angiography. The trial did not meet its primary end point of a reduction of cardiovascular death, MI, or stroke in the overall cohort of ACS patients, and more CURRENT major bleeding was observed with the higher-dose clopidogrel strategy. Lower ischemic event rates were observed with the combination of high-dose clopidogrel and high-dose aspirin (300 to

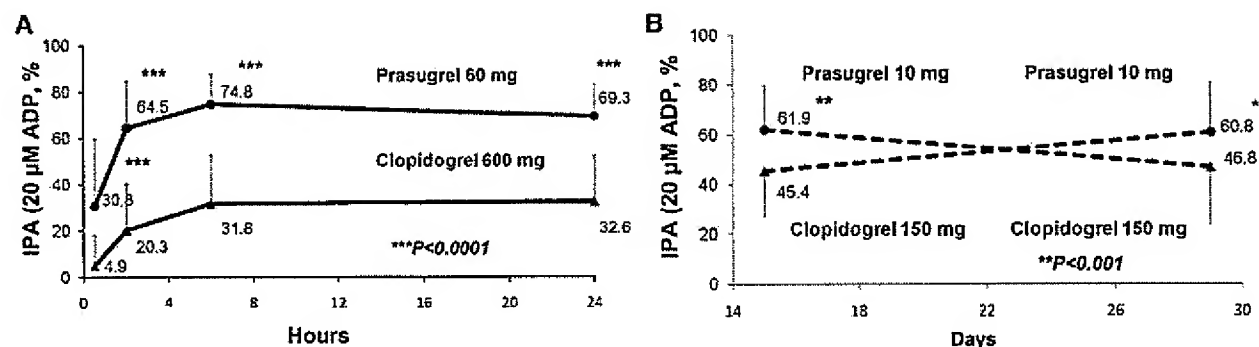


Figure 2. The main results of PRINCIPLE-TIMI 44. Prasugrel 60 mg resulted in higher levels of platelet inhibition than 600 mg clopidogrel in the acute phase, and 10 mg prasugrel resulted in higher levels of platelet inhibition than 150 mg clopidogrel in the chronic phase. IPA indicates inhibition of platelet aggregation. Data derived from Wiviott et al.³⁷

325 mg daily). In a postrandomization subgroup of patients who underwent PCI, there was a reduction of ischemic events, including MI and stent thrombosis, with higher-dose clopidogrel compared with the standard dose.³⁶

The Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation (PRINCIPLE)-TIMI 44 trial compared prasugrel and clopidogrel using the CURRENT OASIS 7 clopidogrel dose regimen (600-mg loading dose followed by 150 mg daily). This was a 2-phase crossover study in patients with coronary artery disease undergoing cardiac catheterization with planned PCI. Prasugrel 60-mg loading dose showed higher levels of platelet inhibition than 600 mg clopidogrel, and 10 mg prasugrel showed greater levels of platelet inhibition than 150 mg clopidogrel daily³⁷ (Figure 2). A significant difference in induced platelet aggregation emerged as soon as 30 minutes and persisted throughout the first 24 hours. In fact, by 30 minutes, the levels of inhibition with prasugrel 60 mg were similar to the maximal inhibition achieved with clopidogrel 600-mg loading dose (Figure 2A). In a crossover design, higher levels of induced platelet aggregation were also observed with 10 mg prasugrel compared with 150 mg clopidogrel after 2 weeks of therapy (Figure 2B).

Clinical Outcomes Trials of Prasugrel

The Joint Utilization of Medications to Block Platelets Optimally (JUMBO)-TIMI 26³⁸ trial was a phase II, dose-ranging, safety study of prasugrel compared with clopidogrel in patients undergoing PCI. In this study, 904 subjects were randomized to 1 of 3 prasugrel dosing strategies with 2 prasugrel loading doses and 3 prasugrel maintenance doses (40/7.5 mg, 60/10 mg, and 60/15 mg) compared with clopidogrel (300/75 mg) and followed up for 30 days. Bleeding rates overall were low, and there were no statistically significant differences among treatment groups for the primary safety end point of TIMI major or minor bleeding. However, numerically more patients had bleeding in the combined prasugrel group (hazard ratio [HR], 1.42; 95% confidence interval [CI] 0.40 to 5.08) compared with clopidogrel, and the 60/15 mg prasugrel arm tended to have higher rates of TIMI minimal bleeding. The study was not powered for efficacy end points, and a nonsignificant difference (HR, 0.76; 95% CI, 0.46 to 1.24) was observed for the primary end point of

major adverse cardiovascular events in favor of prasugrel-treated patients compared with clopidogrel-treated patients. The combination of clinical data from JUMBO-TIMI 26 and platelet function data in healthy volunteers³² and patients³⁹ served as the basis for dose selection for the pivotal phase II, registration pathway trial of prasugrel.

Efficacy

The majority of clinical outcomes data for prasugrel comes from the phase III TRITON-TIMI 38 trial. In this trial, 13 608 subjects with moderate- to high-risk ACS, including UA, NSTEMI, and STEMI with planned PCI, were randomized to receive either clopidogrel 300-mg loading dose followed by 75 mg daily or prasugrel 60-mg loading dose followed by 10 mg daily. Subjects with UA/NSTEMI or STEMI treated initially with medical therapy could be randomized and treated only after the coronary anatomy was known to be suitable for PCI; subjects with STEMI and planned primary PCI could be randomized and treated on first contact. Subjects with recent clopidogrel use, known bleeding diathesis, or other high-risk features for bleeding were excluded.⁴⁰ Subjects were treated for a median of 14.5 months.

The primary end point was the composite of death from cardiovascular causes, nonfatal MI, or nonfatal stroke. Subjects randomized to prasugrel had fewer primary end-point events (9.9% versus 12.1%; HR, 0.81; 95% CI, 0.73 to 0.90; $P<0.001$) compared with clopidogrel, as shown in Figure 3A and Table 1.⁴¹

The primary efficacy end point (Table 1) was driven by a 24% reduction in MI including both fatal and nonfatal MI. Cardiovascular death and total mortality were numerically but not statistically lower (total mortality, 3.0% versus 3.2%; HR, 0.95; 95% CI, 0.78 to 1.16; in STEMI, 3.3% versus 4.3%; HR, 0.76; 95% CI, 0.54 to 1.07; in UA/NSTEMI, 2.9% versus 2.8%; HR, 1.08; 95% CI, 0.84 to 1.38), and no effect was seen on total stroke. Additional analyses of reduction in MI using the American College of Cardiology/American Heart Association/European Society of Cardiology universal MI definition showed similar reductions in procedural MI and spontaneous MI, small and large MIs (based on peak biomarkers), investigator reported MIs, and MIs both early (within 30 days) and after 30 days.⁴² The reduction in clinical ischemic events was also notable for a reduction in urgent target vessel revascularization (2.5% versus 3.7%; HR, 0.66;

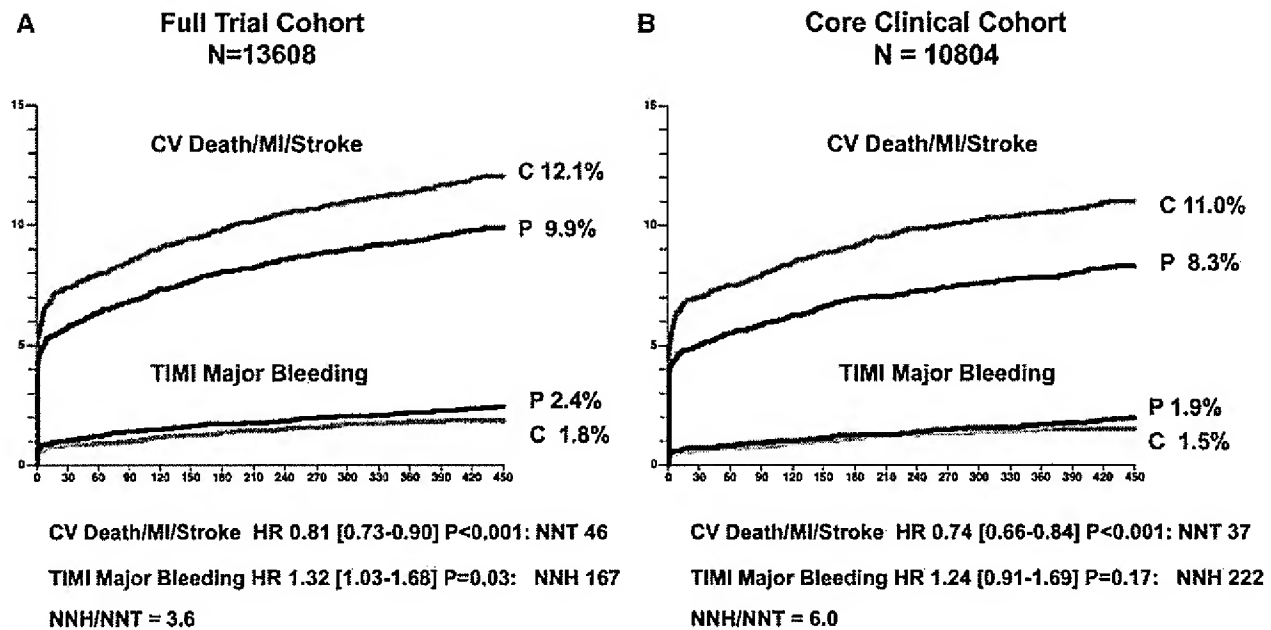


Figure 3. A, TRITON-TIMI 38 main results in the overall cohort. B, Main results figure in core clinical cohort (no history of stroke/transient ischemic attack, age < 75 years, and weight ≥ 60 kg). CV indicates cardiovascular; C, clopidogrel; P, prasugrel; NNT, number needed to treat; NNH, number needed to harm. Data derived from Wiviott et al.⁴¹

95% CI, 0.54 to 0.81; $P < 0.001$). A key finding from TRITON-TIMI 38 was a marked reduction in stent thrombosis among patients receiving prasugrel. Overall for the duration of the trial, Academic Research Consortium-defined definite or probable stent thrombosis was reduced by 52% (1.1% versus 2.4%; HR, 0.48; 95% CI, 0.36 to 0.64; $P < 0.001$) and definite (angiographic or autopsy proven) stent thrombosis by 58% (0.9% versus 2.0%; HR, 0.42; 95% CI, 0.31 to 0.59; $P < 0.001$; Figure 4A)⁴³ in patients who received prasugrel. These findings were similar whether patients received bare metal stents or drug-eluting stents. The reduction in stent thrombosis was also noted both early, ie, within 30 days after randomization (0.64% versus 1.56%; HR, 0.41; 95% CI, 0.29 to 0.59; $P < 0.0001$), and after 30 days (0.49% versus 0.82%; HR, 0.60; 95% CI, 0.37 to 0.97; $P = 0.03$; Figure 4B).

Safety

Consistent with the more potent antiplatelet effects observed with prasugrel in TRITON-TIMI 38, higher rates of bleeding were observed. The key safety end point of non-coronary artery bypass graft (CABG)-related TIMI major bleeding was observed more frequently with prasugrel (2.4% versus 1.8%; HR, 1.32; 95% CI, 1.03 to 1.68; $P = 0.03$; Figure 3A).⁴¹ In addition to the key safety end point of TIMI major bleeding, there was an excess of non-CABG-related TIMI major or minor bleeding (5.0% versus 3.8%; HR, 1.31; 95% CI, 1.11 to 1.56; $P = 0.002$) and bleeding requiring transfusion (4.0% versus 3.0%; HR, 1.34; 95% CI, 1.11 to 1.63; $P < 0.001$). Among non-CABG-related bleeding, the excess was driven predominantly by an increase in spontaneous bleeding (1.6% versus 1.1%; HR, 1.51; 95% CI, 1.09 to 2.08; $P = 0.01$). Instrumented bleeding and bleeding related to trauma were less frequent in both groups, and rates

Table 1. Key Efficacy and Safety Results From TRITON-TIMI 38

End Point	Clopidogrel, %	Prasugrel, %	Absolute Risk Difference, %	HR	95% CI	P
CVD/MI/CVA	12.1	9.9	2.2	0.81	0.73-0.90	< 0.001
MI	9.7	7.4	2.3	0.76	0.67-0.85	< 0.001
Urgent TVR	3.7	2.5	1.2	0.66	0.54-0.81	< 0.001
TIMI major bleeding*	1.8	2.4	0.6	1.32	1.03-1.68	0.03
TIMI major or minor bleeding*	3.8	5.0	1.2	1.31	1.11-1.56	0.002
Bleeding requiring transfusion*	3.0	4.0	1.0	1.34	1.11-1.63	< 0.001
All-cause death, MI, stroke, TIMI major bleeding*	13.9	12.2	1.7	0.87	0.79-0.95	0.004

CVD indicates cardiovascular death; CVA, cerebrovascular accident (stroke); and TVR, target vessel revascularization.

*Not related to coronary artery bypass surgery.

Data derived from Wiviott et al.⁴¹

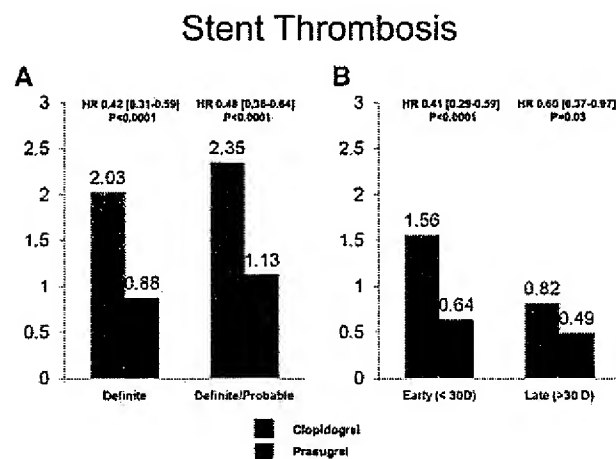


Figure 4. Stent thrombosis in TRITON-TIMI 38. A, Stent thrombosis in the overall cohort for the duration of the trial. B, Academic Research Consortium definite or probable stent thrombosis by timing. Data derived from Wiviott et al.⁴³

were similar between the 2 treatment arms. Among the non-CABG major bleeds, there was an excess of life-threatening bleeding and, though rare, fatal bleeding. Intracranial bleeding was not increased among the patients treated with prasugrel (0.3% of both treatment arms).

The relative bleeding excess with prasugrel tended to be similar among major subgroups and tended to continue to accumulate over time; no significant difference in TIMI major bleeding was observed by 30 days (1.03% versus 0.87%; HR, 1.19; 95% CI, 0.84 to 1.68; $P=0.34$), and bleeding in this time period was most often procedure related with both agents. However, after 30 days, a significant excess in TIMI major bleeding was observed (1.42% versus 0.97%; HR, 1.48; 95% CI, 1.04 to 2.09; $P=0.03$) and was more commonly spontaneous bleeding, particularly gastrointestinal bleeding. No significant interaction between time of follow-up and treatment was observed. Because TRITON-TIMI 38 was designed as a PCI trial in which coronary anatomy had to be known to be suitable for PCI before randomization, CABG was infrequent. TIMI major bleeding was identified in 13% of prasugrel-treated patients who underwent CABG (0.4% of the total cohort) compared with 3% of clopidogrel-treated patients who underwent CABG (0.1% of the total cohort; HR, 4.73; 95% CI, 1.90 to 11.82; $P<0.001$).

To assess the balance between safety and efficacy, a prespecified net outcome end point of all-cause death, MI, stroke, and non-CABG TIMI major bleeding was evaluated in TRITON-TIMI 38. In the overall cohort, this end point favored prasugrel (12.2% versus 13.9%; HR, 0.87; 95% CI, 0.79 to 0.95; $P=0.004$). This net outcome was robust to sensitivity analyses, including less severe bleeding episodes (such as TIMI minor bleeding).⁴⁴ In addition to the prespecified safety end points, a careful assessment of reported adverse events identified a slight excess in patients with new or worsened malignancies, particularly colon cancers, which may have resulted from ascertainment bias caused by earlier identification in the prasugrel group as a result of evaluations for bleeding or anemia. The US Food and Drug Administration determined that there was not sufficient biological evidence to suggest that prasugrel was a carcinogen or a

tumor promoter and requested additional studies to assess the potential cancer risk.⁴⁵

Subgroups

Several subgroups of patients in TRITON-TIMI 38 are of both scientific and clinical interest. Three groups were highlighted as being of particular concern: those with previously known stroke, elderly patients, and patients with low body weight. Patients with a self-reported or known history of stroke or transient ischemic attacks ($n=518$) before enrollment in TRITON-TIMI 38 had a higher rate of primary efficacy events (19.1% versus 14.4%; HR, 1.37; $P=0.15$) driven by an increase in stroke, which differed significantly from the non-stroke cohort (P for interaction=0.02). Coupled with a high rate of bleeding, including more intracranial hemorrhage in this subgroup, the net outcome significantly favored clopidogrel (23.0% versus 16.0%; HR, 1.54; $P=0.04$).

In patients ≥ 75 years of age ($n=1809$), a smaller (but directionally consistent) relative reduction in primary efficacy events (17.2% versus 18.3%; HR, 0.94; $P=0.60$), coupled with higher absolute TIMI major bleeding rates (4.2% versus 3.4%; HR, 1.36; $P=0.24$), resulted in a balance between efficacy and safety and a neutral net outcome (21.7% versus 21.5%; HR, 0.99; $P=0.92$). Of particular concern in the elderly patients was the higher rate of spontaneous fatal hemorrhage compared with younger patients. In patients ≥ 75 years of age, 9 spontaneous fatal hemorrhages were observed with prasugrel and 0 with clopidogrel. In patients <75 years of age, 5 fatal spontaneous hemorrhages were observed with prasugrel and 4 with clopidogrel.

Patients with low body weight (<60 kg; $n=668$), in whom pharmacokinetic modeling demonstrated an overexposure to the prasugrel active metabolite,^{30,41} had an efficacy similar to that of the overall cohort (10.5% versus 12.6%; HR, 0.86; $P=0.54$), had higher rates of bleeding (6.0% versus 3.5%; HR, 1.90; $P=0.09$), and had a neutral net outcome (15.9% versus 15.7%; HR, 1.03; $P=0.89$). It should be recognized that the relative efficacy and safety of prasugrel appear to be optimized in TRITON-TIMI 38 in patients <75 years of age and weighing >60 kg, but it is also likely that the general principle holds that this balance worsens with advancing age and decreasing body weight beyond these precise thresholds (Tables I and II in the online-only Data Supplement).

In contrast, patients with diabetes mellitus had a greater reduction in cardiovascular death/MI/stroke (12.2% versus 17.0%; HR, 0.70; $P<0.001$) than those without diabetes mellitus (9.2% versus 10.6%; HR, 0.86; $P=0.02$; P for interaction=0.09) without an excess in TIMI major bleeding, resulting in a greater improvement in the net outcome.⁴⁶ There were consistent benefits among patients presenting with STEMI⁴⁷ and those with UA/NSTEMI, although there appeared to be less bleeding excess in patients with STEMI. There were consistent benefits of prasugrel compared with clopidogrel among patients who received varying doses of aspirin,⁴⁸ patients with or without glycoprotein IIb/IIIa inhibitors,⁴⁹ and those with or without proton pump inhibitors.⁵⁰

A pharmacokinetic substudy of TRITON-TIMI 38 demonstrated that patient groups who had less favorable outcomes, patients who were either <60 kg or ≥ 75 years of age,

had higher levels of the active metabolite of the drug than did subjects without those characteristics.³⁰ Modeling data suggest that decreasing the maintenance dose of prasugrel to 5 mg in these subjects would reduce exposure to the active metabolite to levels consistent with those observed in the subjects <75 years of age and ≥ 60 kg. The efficacy and safety of the 5-mg doses in this population have not been established in a clinical trial but are being tested in the ongoing Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes (TRILOGY ACS) NCT00699998 trial, which is also testing the efficacy of prasugrel compared with clopidogrel in patients with ACS without intended PCI.

Clinical Genetics

As noted above, for clopidogrel or prasugrel to exert an antiplatelet effect, metabolism from an inactive parent compound to the active metabolite that interacts with the P2Y₁₂ receptor is required (Figure 1). The CYP enzymes involved in these conversions are known to be subject to common genetic variation resulting in differential function. The combination of the need for metabolism for action and the interpatient variability in metabolic efficiency sets the stage for a pharmacogenomic treatment interaction with clopidogrel. Indeed, several studies have reported that patients who are carriers of a reduced-function allele of *CYP 2C19* are at increased risk of recurrent cardiovascular events, including recurrent MI and stent thrombosis, while being treated with clopidogrel.^{24,51–53} In the genetic analysis of TRITON–TIMI 38, $\approx 30\%$ of subjects tested were carriers of at least 1 common reduced-function allele for *CYP 2C19*. Among the clopidogrel subjects, carriers had an excess of cardiovascular ischemic events, including a 3-fold higher rate of stent thrombosis.²⁴ These data were supported by consistent effects of the reduced function alleles of *CYP 2C19* showing less generation of active metabolite and less inhibition of platelets in clopidogrel-treated patients.²⁴ In support of the mechanistic importance of the *CYP 2C19* enzyme, a genome-wide association study identified loci associated with genes encoding *CYP 2C19* associated with reduced antiplatelet effect of clopidogrel.⁵⁴ In contrast to the consistent observations of pharmacogenomic effects on clopidogrel, none of the common variants in the *CYP* genes tested showed consistent reductions in prasugrel active metabolite generation and the antiplatelet effects of prasugrel.²³ Consequently, subjects assigned to prasugrel in the TRITON–TIMI 38 genetic analysis had no difference in the rates of cardiovascular ischemic events by genotype, suggesting that the more efficient metabolism of prasugrel may render patients less susceptible to such genetic variation.²³

Regulatory Action and Anticipated Clinical Use

Largely on the basis of the aforementioned TRITON–TIMI 38 trial, prasugrel received approval by both the US Food and Drug Administration and the European Medicines Agency for use in patients with ACS (including STEMI and UA/NSTEMI) undergoing planned PCI (Table 2). Both agencies provided warnings of the bleeding risk with prasugrel,

including a “black box” warning by the Food and Drug Administration, and provided contraindications for its use in patients with prior stroke or transient ischemic attack. On the basis of regulatory action, the core clinical cohort recommended for treatment with prasugrel at the studied doses (60 mg and 10 mg) included patients without prior stroke or transient ischemic attack who were <75 years of age and weighed ≥ 60 kg. This group of patients, who represented 79.4% of the TRITON–TIMI 38 population, exhibited a greater benefit of prasugrel on ischemic end points and had less absolute bleeding difference (Figure 3B). The American and European regulatory agencies approved both 10- and 5-mg tablets of prasugrel and recommended the 5-mg tablet for patients weighing <60 kg (132 lb). For patients ≥ 75 of age, the US Food and Drug Administration indicated that prasugrel is generally not recommended, but its use may be considered in patients at high risk for recurrent ischemic events such as those with diabetes mellitus or prior MI; it also recommended that when prasugrel is used in the elderly weighing ≥ 60 kg, prasugrel should be used with its standard dosing regimen. The European Medicines Agency took a slightly different approach, also recommending that prasugrel should generally be avoided in the elderly, but if used, the dose should be lowered to 5 mg.

Summary and Recommendations

Prasugrel is a third-generation thienopyridine with more consistent and efficient metabolism than clopidogrel, the current standard of care. Pharmacodynamic studies have shown that patients taking prasugrel compared with standard or higher doses of clopidogrel have higher levels of thienopyridine active metabolite and higher and more consistent levels of platelet inhibition. Prasugrel appears less susceptible to genetic variation and drug-drug interactions, which can limit the antiplatelet activity and clinical effectiveness of clopidogrel. This pharmacokinetic and pharmacodynamic superiority is translated into improved ischemic outcomes but more bleeding in patients with ACS and planned PCI. These data serve as the first to definitively demonstrate that an agent (or dose of an agent) with higher and more consistent levels of platelet P2Y₁₂ inhibition can reduce ischemic events, a key research question in cardiology, and set the stage for further research on personalized medicine, alternative agents, and alternative platelet targets. These data, however, do not indicate the presence of specific goal levels of platelet inhibition for individual patients. Ticagrelor, a direct, non-thienopyridine P2Y₁₂ inhibitor, has also been shown to improve clinical outcomes, including total mortality, in patients with ACS compared with clopidogrel,⁵⁵ further confirmation of the importance of oral platelet inhibition.

From a patient care standpoint, the favorable balance of risk and benefit in a large majority of the patients studied in TRITON–TIMI 38 served as the basis for approval of prasugrel worldwide by regulatory agencies. Prasugrel is now available for prescription by physicians in the United States and in many countries around the world as an alternative to clopidogrel. Available evidence supports the use of prasugrel either in patients with ACS who are undergoing planned primary PCI for STEMI or in patients with UA/NSTEMI or

Table 2. Summary of Regulatory Action Related to Prasugrel

	US Food and Drug Administration	European Medicines Agency
Indication	Reduction of thrombotic cardiovascular events (including stent thrombosis) in patients with ACS who are to be managed with PCI	Prevention of atherothrombotic events in patients with ACS undergoing primary or delayed PCI
Dose and administration	Initiate treatment with a single 60-mg oral loading dose and continue at 10 mg once daily with or without food	Should be initiated with a single 60-mg loading dose and then continued at 10 mg once a day Premature discontinuation of any antiplatelet agent, including prasugrel, could result in an increased risk of thrombosis, MI, or death resulting from the patient's underlying disease Treatment of up to 12 mo is recommended unless the discontinuation of prasugrel is clinically indicated
Contraindications	Active pathological bleeding Prior stroke or transient ischemic attack	Hypersensitivity to the active substance or to any of the excipients Active pathological bleeding History of stroke or transient ischemic attack Severe hepatic impairment (Child Pugh class C)
Warnings and precautions	Can cause significant, sometimes fatal, bleeding Increased risk in patients likely to undergo CABG Premature discontinuation increases the risk of stent thrombosis, MI, and death	Patients with ACS undergoing PCI treated with prasugrel and ASA showed an increased risk of major and minor bleeding; therefore, the use of prasugrel in patients at increased risk of bleeding should be considered only when the benefits in terms of prevention of ischemic events are deemed to outweigh the risk of serious bleedings
Age ≥ 75 y	Generally not recommended except in high-risk patients (diabetes mellitus or prior MI) in whom its effect appears to be greater and its use may be considered	Generally not recommended and should be undertaken only with caution after a careful individual benefit/risk evaluation by the prescribing physician If prescribed, a lower maintenance dose of 5 mg should be used; the 10-mg maintenance dose is not recommended
Weight < 60 kg	Consider 5 mg once daily	A 5-mg maintenance dose should be used
Genetics	No relevant effect of genetic variation	No relevant effect of genetic variation
Drug-drug Interactions	No relevant effects of inducers or inhibitors of CYP enzymes or drugs that interfere with gastric pH	Can be administered concomitantly with drugs that inhibit or induce CYP enzymes and drugs that interfere with gastric pH

ASA indicates acetylsalicylic acid.

STEMI in whom the coronary anatomy is known to be suitable for PCI who have not been treated with clopidogrel.

Prasugrel has not been compared with pretreatment with clopidogrel. In patients with adequate time for clopidogrel pretreatment before catheterization, the relative efficacy of the 2 strategies of treatment is unknown. Because of its rapid onset of action, loading of prasugrel at the time of angiography can be expected to provide high-level platelet inhibition to support PCI and to reduce the risk of early ischemic events such as acute stent thrombosis. Although CABG-related bleeding was more frequent with prasugrel than with clopidogrel, the ability to use prasugrel to support PCI when coronary anatomy is known should reduce the frequency with which patients who are identified as needing CABG will receive a thienopyridine. Therefore, the use of prasugrel appears particularly well suited for patients who will have cardiac catheterization and PCI within hours (rather than days) of the decision to use a thienopyridine and in whom this more rapid agent can be given peri-PCI and will be active at the time of PCI. Compared with standard-dose clopidogrel, prasugrel provides greater protection from ischemic events, particularly stent thrombosis, repeat revascularization, and MI, throughout 15 months in patients with PCI but with more

bleeding. The use of prasugrel appears to have a particularly favorable risk-to-benefit profile in patients with demographic factors that place them at very high risk for recurrent ischemic events but not at high risk for bleeding such as those with ACS and diabetes mellitus and those with STEMI, although clinical trial data do not suggest the limitation of use to these patients.

It may seem logical to extrapolate from previous data to suggest that one may guide therapy on the basis of genetic testing (CYP 2C19 polymorphisms) or to reserve a more potent agent such as prasugrel for patients who are demonstrated to have poor platelet response to clopidogrel on functional testing. However, we do not believe there is currently sufficient evidence or practical availability of rapid testing to identify patients with these particular risk factors. In the set of patients for whom prasugrel is indicated, if physicians wait for the results of genetic testing or initiate clopidogrel and await platelet function testing, the early beneficial effects of rapid and higher level P2Y₁₂ inhibition could be lost.

Therefore, as with the incorporation of other new therapies into clinical practice, prasugrel should be used as one component of a treatment strategy to optimize patient outcomes,

balancing ischemic protection, bleeding risk, and cost. From a demographic standpoint, the balance appears to favor efficacy with prasugrel as studied with the exclusion of a well-defined subgroups of patients, including those with prior stroke or transient ischemic attack, the elderly, or patients of low body weight, without additional risk features for recurrent ischemia and strongly favors efficacy in the setting of diabetes mellitus or STEMI. For individual patients, the clinician must integrate the choice and dose of thienopyridine, invasive strategies, vascular access management, stent types, choice of and dose of additional antiplatelet agents, antithrombins, and adjunctive pharmacotherapies.

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KEY WORDS: anticoagulants ■ platelet aggregation inhibitors ■ platelets ■ thrombosis

Supplemental Tables

Table 1 (Supplemental): Clinical outcomes by weight at time of enrollment. Primary Endpoint = Cardiovascular death/myocardial infarction/stroke. Net Endpoint = All-cause death/myocardial infarction/stroke/TIMI major non-CABG bleed.

			<u>Prasugrel</u>	<u>Clopidogrel</u>		
Endpoint	Weight in Kilograms	N*	Event Rate	Event Rate	Hazard ratio	Interaction p-value
Primary EP (CVD/MI/stroke)	<60	668	10.5%	12.6%	0.86 (0.54-1.38)	0.55
	60- <70	1808	11.0%	13.7%	0.80 (0.61-1.04)	
	70- <80	3278	10.6%	12.6%	0.85 (0.69-1.04)	
	80- <90	3443	9.1%	10.9%	0.80 (0.65-1.00)	
	90- <100	2171	9.6%	11.1%	0.85 (0.65-1.11)	
	>=100 kg	2069	8.2%	11.2%	0.72 (0.54-0.96)	
TIMI major non-CABG bleed	<60	664	6.0%	3.5%	1.90 (0.90-4.02)	0.92
	60- <70	1791	2.1%	2.3%	0.96 (0.50-1.85)	
	70- <80	3255	2.2%	2.0%	1.24 (0.76-2.04)	
	80- <90	3420	2.6%	1.7%	1.41 (0.86-2.33)	
	90- <100	2154	1.5%	1.6%	0.96 (0.47-1.96)	
	>=100 kg	2052	2.3%	1.2%	1.75 (0.83-3.68)	
Net endpoint	<60	668	15.7%	15.9%	1.03 (0.69-1.53)	0.83
	60- <70	1808	13.2%	16.0%	0.81 (0.63-1.03)	
	70- <80	3278	12.5%	14.4%	0.87 (0.72-1.06)	
	80- <90	3443	11.3%	12.6%	0.86 (0.71-1.06)	
	90- <100	2171	11.8%	12.4%	0.93 (0.73-1.20)	
	>=100 kg	2069	10.8%	12.8%	0.82 (0.63-1.06)	

*Subjects for whom weight was not known at the time of enrollment are excluded from the analysis. N represents the intention to treat cohort for the primary and net endpoints and the safety cohort for TIMI major non-CABG bleeding

Table 2 (Supplemental): Clinical outcomes by age at time of enrollment. Primary Endpoint = Cardiovascular death/myocardial infarction/stroke. Net Endpoint = All-cause death/myocardial infarction/stroke/TIMI major non-CABG bleed.

			Prasugrel	Clopidogrel		
	Age in years	N*	Event Rate	Event Rate	Hazard ratio	Interaction p-value
Primary EP	<45	974	6.2%	11.0%	0.54 (0.34-0.86)	0.01
	45-49	1332	6.2%	9.3%	0.68 (0.45-1.01)	
	50-54	1902	6.7%	9.8%	0.69 (0.50-0.95)	
	55-59	2217	9.5%	11.6%	0.81 (0.62-1.05)	
	60-64	1897	9.9%	10.9%	0.88 (0.66-1.17)	
	65-69	1919	9.4%	11.0%	0.82 (0.62-1.09)	
	70-74	1558	12.2%	13.9%	0.91 (0.68-1.20)	
	>=75	1809	17.2%	18.3%	0.94 (0.75-1.18)	
TIMI major non-CABG bleed	<45	963	0.7%	1.0%	0.81 (0.18-3.61)	0.84
	45-49	1315	2.1%	0.8%	2.48 (0.88-6.96)	
	50-54	1890	1.8%	1.2%	1.47 (0.66-3.28)	
	55-59	2194	1.9%	1.8%	0.95 (0.49-1.85)	
	60-64	1883	2.8%	1.8%	1.57 (0.82-2.98)	
	65-69	1891	2.7%	2.3%	1.17 (0.64-2.12)	
	70-74	1536	2.6%	1.8%	1.35 (0.66-2.78)	
	>=75	1785	4.2%	3.4%	1.36 (0.81-2.27)	
Net endpoint	<45	974	6.7%	12.0%	0.54 (0.34-0.84)	0.02
	45-49	1332	8.9%	9.8%	0.89 (0.62-1.29)	
	50-54	1902	7.8%	10.8%	0.71 (0.53-0.97)	
	55-59	2217	11.5%	13.6%	0.83 (0.65-1.07)	
	60-64	1897	12.5%	12.3%	0.97 (0.75-1.26)	
	65-69	1919	12.0%	12.9%	0.90 (0.70-1.17)	
	70-74	1558	14.4%	16.5%	0.89 (0.68-1.15)	
	>=75	1809	21.5%	21.7%	0.99 (0.81-1.21)	

*N represents the intention to treat cohort for the primary and net endpoints and the safety cohort for TIMI major non-CABG bleeding

Exhibit 38



Genetic variants in *ABCB1* and *CYP2C19* and cardiovascular outcomes after treatment with clopidogrel and prasugrel in the TRITON-TIMI 38 trial: a pharmacogenetic analysis

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Summary

Background Clopidogrel and prasugrel are subject to efflux via P-glycoprotein (encoded by *ABCB1*, also known as *MDR1*). *ABCB1* polymorphisms, particularly 3435C→T, may affect drug transport and efficacy. We aimed to assess the effect of this polymorphism by itself and alongside variants in *CYP2C19* on cardiovascular outcomes in patients treated with clopidogrel or prasugrel in TRITON-TIMI 38. We also assessed the effect of genotype on the pharmacodynamic and pharmacokinetic properties of these drugs in healthy individuals.

Methods We genotyped *ABCB1* in 2932 patients with acute coronary syndromes undergoing percutaneous intervention who were treated with clopidogrel (n=1471) or prasugrel (n=1461) in the TRITON-TIMI 38 trial. We evaluated the association between *ABCB1* 3435C→T and rates of the primary efficacy endpoint (cardiovascular death, myocardial infarction, or stroke) until 15 months. We then assessed the combined effect of *ABCB1* 3435C→T genotype and reduced-function alleles of *CYP2C19*. 321 healthy individuals were also genotyped, and we tested the association of genetic variants with reduction in maximum platelet aggregation and plasma concentrations of active drug metabolites.

Findings In patients treated with clopidogrel, *ABCB1* 3435C→T genotype was significantly associated with the risk of cardiovascular death, myocardial infarction, or stroke (p=0.0064). TT homozygotes had a 72% increased risk of the primary endpoint compared with CT/CC individuals (Kaplan-Meier event rates 12.9% [52 of 414] vs 7.8% [80 of 1057 participants]; HR 1.72, 95% CI 1.22–2.44, p=0.002). *ABCB1* 3435C→T and *CYP2C19* genotypes were significant, independent predictors of the primary endpoint, and 681 (47%) of the 1454 genotyped patients taking clopidogrel who were either *CYP2C19* reduced-function allele carriers, *ABCB1* 3435 TT homozygotes, or both were at increased risk of the primary endpoint (HR 1.97, 95% CI 1.38–2.82, p=0.0002). In healthy participants, 3435 TT homozygotes had an absolute reduction in maximum platelet aggregation with clopidogrel that was 7.3 percentage points less than for CT/CC individuals (p=0.0127). *ABCB1* genotypes were not significantly associated with clinical or pharmacological outcomes in patients with an acute coronary syndrome or healthy individuals treated with prasugrel, respectively.

Interpretation Individuals with the *ABCB1* 3435 TT genotype have reduced platelet inhibition and are at increased risk of recurrent ischaemic events during clopidogrel treatment. In patients with acute coronary syndromes who have undergone percutaneous intervention, when both *ABCB1* and *CYP2C19* are taken into account, nearly half of the population carries a genotype associated with increased risk of major adverse cardiovascular events while on standard doses of clopidogrel.

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Introduction

In patients presenting with acute coronary syndromes and in those undergoing percutaneous coronary interventions with stenting, dual antiplatelet treatment with aspirin and the thienopyridine clopidogrel is the guideline-approved standard of care.^{1,2} As such, clopidogrel is one of the most frequently prescribed drugs worldwide. However, the pharmacodynamic response to clopidogrel varies substantially between patients,³ and individuals with low platelet inhibition during treatment with clopidogrel are at increased risk of cardiovascular events.⁴ Prasugrel is a third-generation thienopyridine that achieves greater platelet inhibition with less variability between patients than does clopidogrel.⁵ In the Trial to Assess Improvement in

Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction (TRITON-TIMI) 38, treatment with prasugrel compared with clopidogrel resulted in a significantly lower rate of ischaemic events and more bleeding.⁶

Both clopidogrel and prasugrel are prodrugs that need intestinal absorption and subsequent biotransformation to active metabolites by cytochrome P450 enzymes. In several studies, reduced-function genetic variants in *CYP2C19* (located on chromosome 10) have been associated with reduced concentrations of active drug metabolite, diminished platelet inhibition, and higher rates of adverse cardiovascular events in the setting of treatment with clopidogrel, but not prasugrel.^{7–15} To that end, the US Food and Drug Administration has

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incorporated *CYP2C19* genetic information into the updated clopidogrel label in the form of a boxed warning noting that carriers of two reduced-function *CYP2C19* alleles have a diminished response to standard doses of clopidogrel.

Additionally, a key protein involved in thienopyridine absorption is the efflux pump P-glycoprotein, which is encoded by *ABCB1* (also known as *MDR1*, located on chromosome 7). P-glycoprotein is an ATP-dependent efflux pump that transports various molecules across extracellular and intracellular membranes. It is expressed, among other places, on intestinal epithelial cells, where increased expression or function can affect bioavailability of drugs that are substrates. Previous research suggests that when treated with clopidogrel, individuals with genetic variants in *ABCB1* (specifically those who are TT homozygotes for the 3435C→T variant) have reduced concentrations of the active drug metabolite⁸ and increased rates of adverse clinical outcomes.¹⁰ Further investigation into the effect of this polymorphism on outcomes in patients treated with clopidogrel, the effect in relation to *CYP2C19* reduced-function variants, and the effect in those treated with the third-generation thienopyridine prasugrel is needed.

We genotyped a subset of patients in the TRITON-TIMI 38 trial who provided samples for genetic analysis with the aim of assessing the association between the *ABCB1* 3435C→T polymorphism and adverse cardiovascular outcomes during treatment with clopidogrel or prasugrel. To obtain supporting pharmacological data, *ABCB1* genotyping was also done in healthy individuals in whom platelet inhibition and drug concentrations were measured in response to clopidogrel or prasugrel. We also assessed the contribution of the *ABCB1* 3435C→T polymorphism in the context of *CYP2C19* status to elucidate the independent contribution of variants in these two genes.

Methods

Patients

The design and primary results of the TRITON-TIMI 38 trial have been described previously.⁶ Patients with acute coronary syndromes undergoing planned percutaneous coronary interventions were randomly allocated to treatment with clopidogrel (300 mg loading dose followed by 75 mg daily) or prasugrel (60 mg loading dose followed by 10 mg daily) for up to 15 months. We undertook this pharmacogenetic analysis in a TRITON-TIMI 38 genetic substudy that included 2932 patients who both provided a genetic sample and had *ABCB1* genotyped (n=1471 for clopidogrel and n=1461 for prasugrel). This study was approved by institutional review boards, and written informed consent was obtained from all participants.

Healthy participants in seven studies (n=321) involving treatment with clopidogrel or prasugrel, or both, were included in the pharmacodynamic and pharmacokinetic analyses (webappendix pp 1 and 4).⁸

These studies were approved by institutional review boards, and written informed consent was obtained from all participants.

Procedures

In the TRITON-TIMI 38 study, the prespecified primary efficacy endpoint was a composite of cardiovascular death, myocardial infarction, or stroke.⁶ A secondary endpoint was definite or probable stent thrombosis as defined by the Academic Research Consortium.¹⁷ Safety endpoints included TIMI major or minor bleeding not related to coronary artery bypass grafting. These outcomes were adjudicated by a clinical events committee unaware of treatment assignment.

Among the healthy participants, pharmacodynamic response was assessed by use of light transmission aggregometry in response to 20 µmol/L ADP, and was expressed as absolute reduction in maximum platelet aggregation from baseline to 4 h. Plasma concentrations of clopidogrel and prasugrel active drug metabolite were measured by liquid chromatography with mass spectrometry.¹⁸ The area under the plasma concentration-time curve was analysed by the log-linear trapezoidal method from time of dose to the 4-h measurable concentration (AUC₀₋₄).

Genotyping for *ABCB1* was completed with the Affymetrix Targeted Human DMET 1.0 Assay (Affymetrix, Santa Clara, CA, USA) and Illumina Infinium Beadchip Assay (Illumina, San Diego, CA, USA) to minimise missing data.¹⁹ On the basis of previous studies,^{10,16} the main variant of interest was 3435C→T (rs1045642), and participants were classified as homozygous for the C allele (CC), heterozygous (CT), or homozygous for the T allele (TT). Since some in-vitro studies have also assessed a haplotype consisting of 3435C→T and two other *ABCB1* variants, 2677G→T/A (rs2032582) and 1236C→T (rs1128503), we also genotyped these polymorphisms (webappendix p 1).²⁰ Genotypes were in Hardy-Weinberg equilibrium (webappendix p 5).

Because genetic variation in *CYP2C19* has been associated with pharmacological response and cardiovascular outcomes in patients taking clopidogrel,^{8-14,21-24} we assessed the combined effect of genetic variants in *CYP2C19* and *ABCB1* 3435C→T. For *CYP2C19*, participants were genotyped and divided into two groups on the basis of whether they had at least one reduced-function allele (termed carriers) or no reduced-function alleles (termed non-carriers).⁸

Statistical analysis

Analyses were done with SAS (version 9.1) and S-PLUS (version 8.0). On the basis of previous studies, the primary objective was to investigate the association between *ABCB1* 3435C→T genotypes and rates of the primary efficacy endpoint in patients in the TRITON-TIMI 38 study. For consistency with the main trial analyses, the Gehan-

Wilcoxon test was used for the primary efficacy endpoint and log-rank for other endpoints. Event rates were expressed as Kaplan-Meier estimates at 15 months.²⁵ Hazard ratios and 95% CIs were calculated on the basis of Cox proportional hazards regression models with clinical syndrome (non-ST-elevation vs ST-elevation acute coronary syndromes) as a stratification factor. Two-sided p values were calculated to test for differences in cardiovascular event rates between patients stratified by genotype. If a significant association for the primary efficacy evaluation was identified in patients treated with clopidogrel, additional efficacy endpoints were also tested, including the hazards for the components of the composite primary endpoint, the primary endpoint at 30 days, and stent thrombosis. In terms of safety endpoints, TIMI major or minor bleeding not related to coronary artery bypass grafting was assessed until 15 months. Parallel analyses were done for patients allocated treatment with prasugrel.

To elucidate further the contribution of *ABCB1* variants, the associations between additional *ABCB1* genotypes (2677G→T/A, 1236C→T, and the haplotype that included 1236C→T, 2677G→T/A, and 3435C→T; webappendix p 1) and cardiovascular outcomes were tested in each treatment group, with the same methods. We then evaluated 3435C→T in the context of *CYP2C19*. We created Cox proportional hazards regression models examining 3435C→T that were adjusted for *CYP2C19* reduced-function allele status as well as models that stratified patients into four groups on the basis of 3435C→T genotype and *CYP2C19* reduced-function allele status. We did a meta-analysis that included results from FAST-MI[®] by combining HRs for each study using a fixed-effects model with weighting based on inverse variance.

We tested the associations between genetic variation and pharmacodynamic and pharmacokinetic parameters using likelihood ratio tests based on linear regression or mixed-effects models. The primary outcomes were platelet inhibition (change in maximum platelet aggregation) and exposure to active drug metabolite [$\log(\text{AUC}_{0-\infty})$]. The models contained subject as a random effect when repeated measures were present, genotype as the predictor of main interest, and other fixed effects including study, dose, and ethnic origin, and for pharmacodynamics, maximum platelet aggregation at baseline. Other demographic variables, including bodyweight, age, sex, and smoking, were included as judged to be appropriate for each drug, as has been done previously.⁸ Additional models were also created with adjustment for *CYP2C19*.

Role of the funding source

The TRITON-TIMI 38 genetic study was designed and undertaken in collaboration between the TIMI Study Group and the sponsors. The academic authors directed and had access to all the analyses and the full clinical database, wrote all drafts of the report, decided to publish

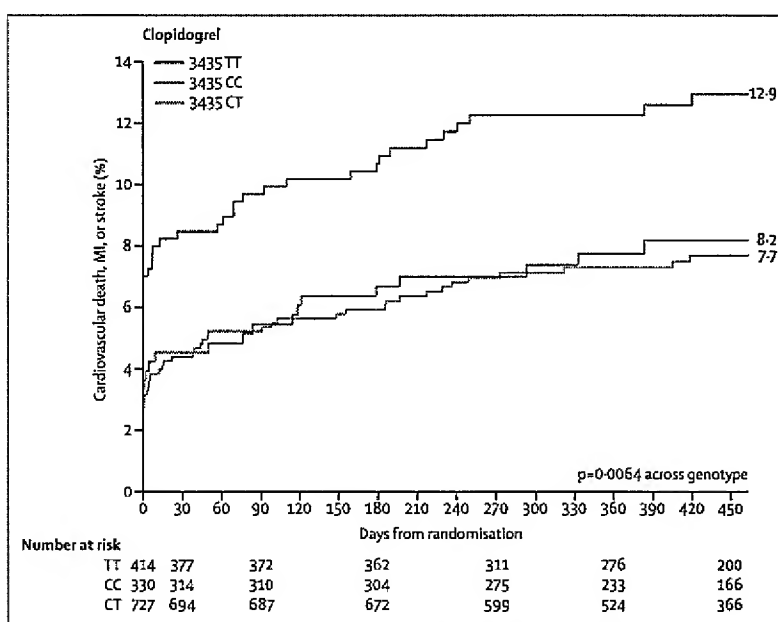


Figure 1: *ABCB1* 3435C→T and cardiovascular outcomes in patients treated with clopidogrel. Cumulative risk of cardiovascular death, myocardial infarction (MI), or stroke for each genotype, with a p value across genotype.

the results, and vouch for the accuracy and completeness of the data.

Results

For the 2932 patients in the TRITON-TIMI 38 genetic substudy, the average age was 60.2 (SD 10.9) years, 831 (28%) were women, 2064 (70%) presented with non-ST-elevation acute coronary syndromes, and 868 (30%) presented with ST-elevation myocardial infarction. For *ABCB1* 3435C→T, 804 (27%) participants in the genetic study population were TT homozygotes, 1459 (50%) CT heterozygotes, and 669 (23%) CC homozygotes. Baseline characteristics in the TRITON-TIMI 38 trial by 3435C→T genotype are shown in the webappendix p 6.

In patients in the TRITON-TIMI 38 genetic substudy who were allocated to treatment with clopidogrel ($n=1471$), 3435C→T genotype was significantly associated with risk of the primary endpoint of cardiovascular death, myocardial infarction, or stroke ($p=0.0064$; figure 1). TT homozygotes were at significantly increased risk compared with CC individuals (HR 1.69, 95% CI 1.05–2.72); CT heterozygotes were at similar risk to CC individuals (HR 0.94, 0.58–1.51). Thus, TT homozygotes for 3435C→T had a 72% increased risk of the primary endpoint compared with CT/CC individuals (Kaplan-Meier event rates 12.9% [52 of 414] vs 7.8% [80 of 1057 participants]; HR 1.72, 95% CI 1.22–2.44, $p=0.002$) when assessed until 15 months. Among 3435 TT versus CT/CC patients, the HR for cardiovascular death was 1.63 (Kaplan-Meier event rates 1.3% [five of 414] vs 0.9% [eight of 1057]; 95% CI 0.53–4.98, $p=0.388$), that for non-fatal myocardial infarction was 1.82 (12.0% [48 of 414] vs 6.8% [70 of 1057]; 1.26–2.62, $p=0.0013$), and that

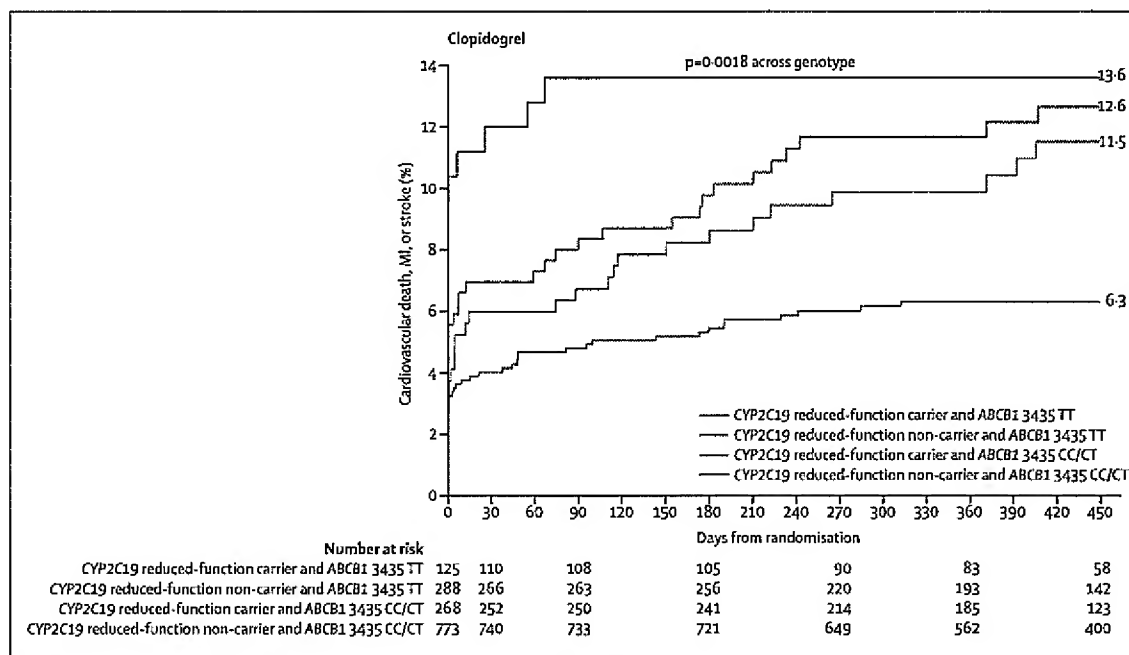


Figure 2: *ABCB1* 3435C→T, *CYP2C19*, and cardiovascular outcomes in patients treated with clopidogrel
Cumulative risk of cardiovascular death, myocardial infarction (MI), or stroke for the four genotype categories, with the p value across genotype category.

for non-fatal stroke was 1.66 (0.6% [two of 414] vs 0.3% [three of 1057 participants]; 0.28–9.93, $p=0.575$). Moreover, the increased risk was evident by 30 days, by which time the risk of the primary endpoint for the 3435 TT homozygotes was roughly twice as high as that for CT/CC individuals (Kaplan-Meier event rates 8.5% [35 of 414] vs 4.5% [47 of 1057 participants]; HR 1.96, 95% CI 1.26–3.03, $p=0.0022$).

Rates of stent thrombosis did not differ significantly between 3435 TT and CT/CC individuals (Kaplan-Meier event rates 1.3% [five of 396] vs 1.3% [12 of 1004 participants]; HR 1.07, 95% CI 0.38–3.04, $p=0.9$). Rates of TIMI major or minor bleeding not related to coronary artery bypass grafting did not differ significantly by genotype (Kaplan-Meier event rates 3.6% [15 of 414] TT homozygotes vs 2.5% [26 of 1052] CT/CC individuals; HR 1.49, 95% CI 0.79–2.82, $p=0.214$).

To elucidate further the contribution of *ABCB1* variants in the setting of treatment with clopidogrel, two other *ABCB1* polymorphisms were explored: 2677G→T/A and 1236C→T. Neither variant was significantly associated with risk of cardiovascular death, myocardial infarction, or stroke (webappendix pp 2, 7, and 8). We constructed haplotypes using the three loci (1236C→T, 2677G→T/A, and 3435C→T) and did not identify associations beyond what was seen for 3435C→T alone (webappendix p 2).

In a model containing both *ABCB1* 3435C→T genotype and *CYP2C19* reduced-function allele carrier status in patients in the TRITON-TIMI 38 genetic substudy treated with clopidogrel, both variants were significant, independent predictors of cardiovascular death, myocardial

infarction, or stroke (*ABCB1* 3435 TT vs CT/CC, HR 2.01, 95% CI 1.30–3.11, $p=0.0017$; *CYP2C19* reduced-function allele carrier vs non-carrier, HR 1.77, 1.11–2.80, $p=0.0155$). When the participants were divided into four groups on the basis of *ABCB1* 3435C→T genotype and *CYP2C19* status (figure 2), the 773 patients (53% of 1454 genotyped) who did not carry at-risk genotypes in either gene had a low rate of cardiovascular death, myocardial infarction, or stroke at 15 months (Kaplan-Meier event rate 6.3%, 48 of 773 participants). By contrast, event rates were significantly higher in the 681 patients (47% of 1454 genotyped) who were either carriers of a *CYP2C19* reduced-function allele only (Kaplan-Meier event rate 11.5%, 29 of 268 participants), *ABCB1* 3435 TT homozygotes only (Kaplan-Meier event rate 12.6%, 35 of 288 participants), or both (Kaplan-Meier event rate 13.6%, 17 of 125 participants) (pooled HR 1.97, 95% CI 1.38–2.82, $p=0.0002$).

When we examined the early timepoint of 30 days, individuals who did not carry either at-risk variant were at low risk (Kaplan-Meier event rate 4.0%, 31 of 773 participants), those who were either *ABCB1* 3435 TT homozygotes or carriers of a *CYP2C19* reduced-function allele were at intermediate risk (Kaplan-Meier event rate 7.0% [20 of 288 participants] for TT homozygotes and 6.0% [16 of 268 participants] for carriers of a reduced-function allele; pooled HR 1.64, 95% CI 1.01–2.65, $p=0.0441$ vs carriers of neither), and individuals who were both *CYP2C19* reduced-function allele carriers and *ABCB1* 3435 TT homozygotes were at high risk (Kaplan-Meier event rate 12.0%, 15 of 125 participants; HR 3.16, 95% CI 1.71–5.85, $p=0.0003$ vs carriers of neither).

There was no significant association between *ABCB1* 3435C→T genotype and risk of cardiovascular death, myocardial infarction, or stroke among patients in the TRITON-TIMI 38 genetic substudy who had been allocated to prasugrel (*n*=1461; figure 3 and webappendix p 2). Specifically, TT homozygotes did not have a significantly higher risk of the primary efficacy endpoint of cardiovascular death, myocardial infarction, or stroke than did CT/CC carriers (Kaplan-Meier event rates 11.0% [41 of 390] vs 8.7% [91 of 1071 participants]; HR 1.25, 95% CI 0.86–1.81, *p*=0.235) when assessed until 15 months. Rates of TIMI major or minor bleeding not related to coronary artery bypass grafting did not differ significantly by *ABCB1* 3435C→T genotype (webappendix p 2). In terms of other *ABCB1* variants, the 2677G→T/A and 1236C→T genotypes overall were not significantly associated with risk of the primary efficacy endpoint in patients treated with prasugrel, although there was a non-significant trend for 2677 TT homozygotes versus CT/CC individuals to be at increased risk (Kaplan-Meier event rates 11.8% [29 of 253] vs 8.8% [94 of 1104 participants]; HR 1.38, 95% CI 0.91–2.09, *p*=0.1290; webappendix pp 3, 9, and 10). When we divided patients on the basis of *ABCB1* 3435C→T genotypes and *CYP2C19* status, rates of cardiovascular death, myocardial infarction, or stroke at 15 months were similar in the four groups (*p*=0.4851, figure 4).

In healthy participants treated with clopidogrel, *ABCB1* 3435 TT homozygotes had a diminished pharmacodynamic effect, with an absolute reduction in maximum platelet aggregation in response to a clopidogrel loading dose that was 7.3 percentage points lower (ie, less platelet inhibition) than that seen in CT/CC individuals (*p*=0.0127). After adjustment for *CYP2C19* genotype, the response was 6.6 percentage points lower (*p*=0.022). The pharmacodynamic effects of clopidogrel on TT carriers were discernible only after a loading dose (for both 300 mg and 600 mg); no significant association was identified during maintenance dosing. There was no significant association between 3435C→T genotype and exposure to clopidogrel active metabolite concentrations. In prasugrel-treated individuals, 3435C→T genotype was not significantly associated with platelet response (1.3 percentage points higher; *p*=0.4345) or exposure to prasugrel's active metabolite. There was no relation between genotype status for either 2677G→T/A or 1236C→T and pharmacodynamic outcomes in patients treated with clopidogrel or prasugrel.

Discussion

The pharmacological and clinical response to clopidogrel varies widely between patients, and genetic variants in *CYP2C19* have been shown to affect the response. P-glycoprotein is important in drug transport, and pharmacogenetic interactions with various classes of drugs have been suggested.¹⁶ Our findings show that TT

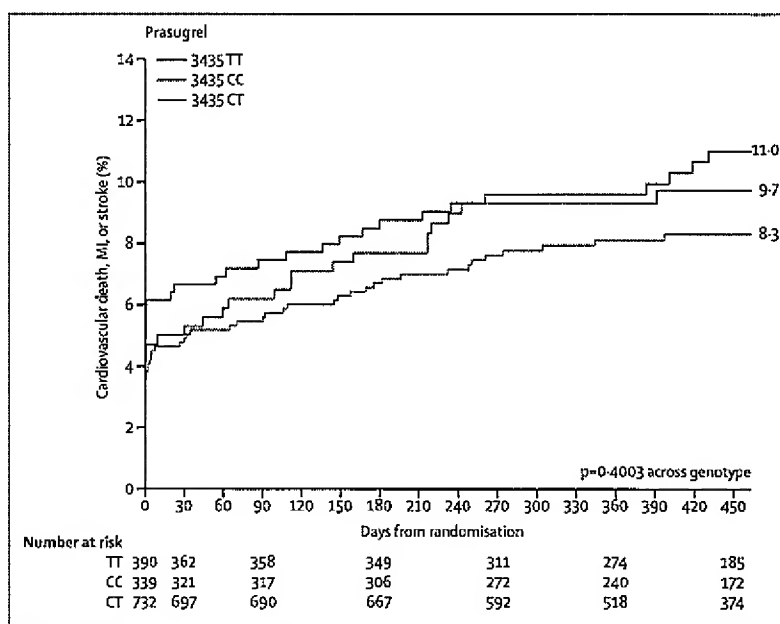


Figure 3: *ABCB1* 3435C→T and cardiovascular outcomes in patients treated with prasugrel. Cumulative risk of cardiovascular death, myocardial infarction (MI), or stroke for each genotype, with a *p* value across genotype.

homozygotes for the 3435C→T variant in *ABCB1* (27% of the study population), as compared with CT/CC individuals, had reduced platelet inhibition with a clopidogrel loading dose in a healthy study population and a significantly increased risk of adverse cardiovascular events during treatment with clopidogrel in patients with acute coronary syndromes undergoing percutaneous coronary intervention. When we considered *ABCB1* 3435C→T genotype in the context of *CYP2C19* reduced-function allele status in patients treated with clopidogrel, we showed that variants in the two genes offered significant, independent information about the risk of cardiovascular death, myocardial infarction, or stroke. Conversely, there were no significant associations between the *ABCB1* variants tested and the response to prasugrel.

ABCB1 encodes the P-glycoprotein efflux transporter. Clopidogrel is a P-glycoprotein substrate, and inhibition of P-glycoprotein affects the bioavailability of clopidogrel.¹⁶ The 3435C→T variant in *ABCB1* is one of the most studied polymorphisms in pharmacogenetic research, and has been associated with altered disposition of several drugs.¹⁶ Although a genome-wide association study identified only *CYP2C19* as being associated with the pharmacodynamic response to clopidogrel, that study showed that platelet response to clopidogrel was highly heritable and was not entirely accounted for by *CYP2C19* status, suggesting that additional genetic variants might be relevant. In a study of patients treated with clopidogrel after elective percutaneous coronary intervention, 3435 TT homozygotes had significantly lower active clopidogrel metabolite concentrations than did CT/CC

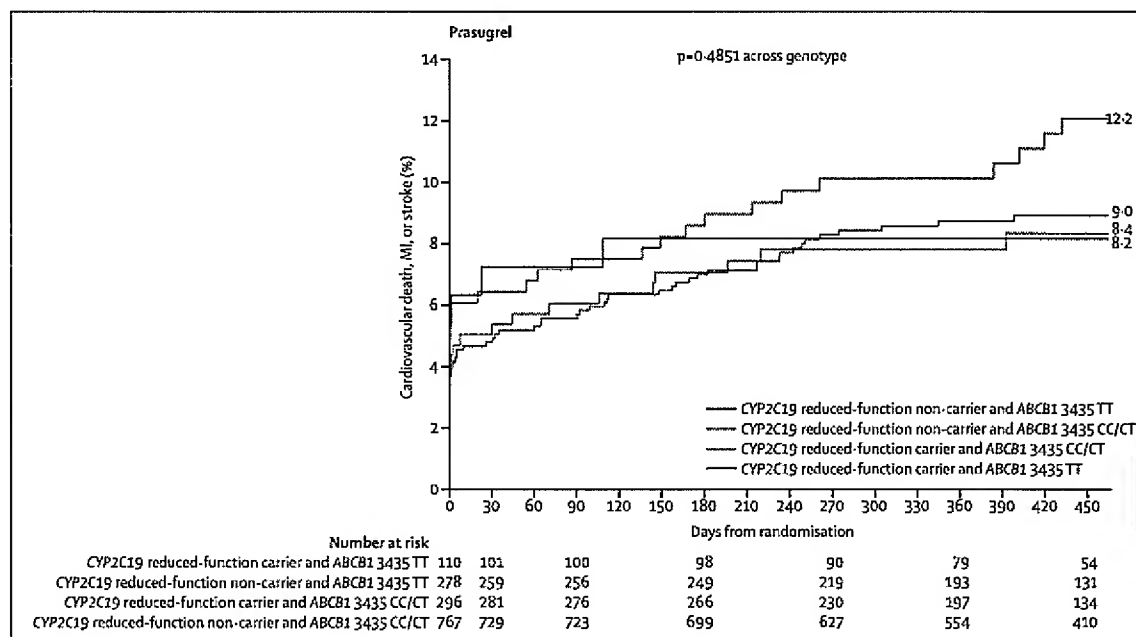


Figure 4: *ABCB1* 3435C→T, *CYP2C19*, and cardiovascular outcomes in patients treated with prasugrel. Cumulative risk of cardiovascular death, myocardial infarction (MI), or stroke for the four genotype categories, with the p value across genotype category.

individuals, suggesting increased intestinal efflux possibly mediated by higher P-glycoprotein expression associated with the 3435 TT genotype.¹⁶ Although evidence on P-glycoprotein expression and activity is inconsistent, mRNA expression in duodenal enterocytes has been reported to be two-to-three-times higher for the *ABCB1* 3435 TT genotype than for either the CC or CT genotype.^{27–29} In the healthy participants in our analysis, TT homozygotes had an absolute reduction in maximum platelet aggregation after a loading dose of clopidogrel that was 7.3 percentage points lower (ie, decreased platelet inhibition) than in CC or CT individuals. Although we did not record a significant association between 3435C→T genotype and pharmacokinetic data, other researchers have shown this relation, and the differences in results could be related to patients, methods, and single-centre versus multicentre study design.

In terms of clinical outcomes, we showed that *ABCB1* 3435 TT homozygotes had a 72% increased risk of adverse cardiovascular events compared with CT/CC individuals in the setting of treatment with clopidogrel in TRITON-TIMI 38. Likewise, in a previous study in patients receiving clopidogrel after an acute myocardial infarction, those who were 3435 TT homozygotes had an increase of about 70% in cardiovascular events during follow-up.¹⁶ In that previous study, however, 3435 CT heterozygotes were also at increased risk of adverse cardiovascular events, albeit less so than were TT homozygotes; the differences in the findings could be attributable to the patient populations. Combination of the results of the previous study and our findings yielded an apparent graded allele-dose response

with an HR for adverse cardiovascular events of 1.29 (95% CI 0.99–1.69) for 3435 CT versus CC individuals and a HR of 1.70 (1.28–2.26) for 3435 TT versus CC individuals. Incorporation of clinical data from other studies will be helpful to further refine the risk estimates. In our analysis, 2677G→T/A and 1236C→T genotypes did not add additional significant information. Nonetheless, further basic genetic pharmacology studies could be helpful to further define the actual functional *ABCB1* variants and the most appropriate genetic model with respect to response to clopidogrel.

In our study, assessment of the contribution of *ABCB1* variants in the context of *CYP2C19* showed that variants in the two genes offered complementary information about cardiovascular risk. When we divided patients into four groups on the basis of *ABCB1* 3435C→T and *CYP2C19* reduced-function allele status, rates of cardiovascular death, myocardial infarction, or stroke until 15 months were nearly twice as high in the study population who were either carriers of a *CYP2C19* reduced-function allele, 3435 TT homozygotes, or both, compared with individuals who did not carry either. Moreover, when both *ABCB1* and *CYP2C19* were taken into account, in this population of patients with an acute coronary syndrome undergoing percutaneous coronary intervention, nearly half of the population carried a genotype associated with increased risk of major adverse cardiovascular events during treatment with standard doses of clopidogrel.

In patients taking prasugrel in TRITON-TIMI 38, *ABCB1* 3435C→T polymorphisms were not significantly associated with cardiovascular outcomes. Likewise, in

healthy participants, no associations between the 3435C→T variant and pharmacokinetic and pharmacodynamic outcomes were seen with prasugrel. The rapid metabolism of prasugrel might mitigate the genetic effect of *ABCB1* 3435C→T polymorphisms, even though the drug is subject to the P-glycoprotein system. Among participants treated with prasugrel, there was a non-significant trend towards 2677 TT homozygotes having higher rates of adverse cardiovascular events compared with the rest of the population. No association was seen with 2677G→T/A and the pharmacological data. Future studies will assist in further examination of these exploratory findings.

There are several limitations to this analysis. First, few non-Caucasian individuals were included in these studies, and future investigations in other populations would be useful. Second, because of the sample handling and the need for repeat measurements for the pharmacokinetic and pharmacodynamic assessments, these investigations were done in healthy individuals, not in the acute clinical trial study population. Third, in our clinical outcomes study, patients treated with clopidogrel received a 300 mg loading dose and 75 mg daily maintenance dose, and patients treated with prasugrel received a 60 mg loading dose and 10 mg daily maintenance dose; we cannot comment on the effect of *ABCB1* genetic variants in patients receiving other doses of these drugs. Fourth, the number of bleeding and stent thrombosis events was small, and our analysis had restricted power to detect an association between the tested *ABCB1* variants and these outcomes. Additional studies that include more such events will be particularly important to further elucidate the relations between *ABCB1* genetic variants and outcomes. Finally, there might be other genetic variants affecting the association between treatment with clopidogrel and cardiovascular outcomes.

In conclusion, we found that *ABCB1* 3435 TT homozygotes had an increased risk of adverse cardiovascular outcomes during treatment with clopidogrel after an acute coronary syndrome and percutaneous coronary intervention. Thus, the association between *ABCB1* polymorphisms and ischaemic risk in patients treated with clopidogrel has been noted now in several pharmacological and clinical outcomes studies. Our analysis also shows that the pharmacogenetic effects of *ABCB1* 3435C→T are independent of and complementary to those of *CYP2C19*. As clinicians, professional societies, and patients integrate information about genetic factors affecting the response to thienopyridines, the roles of both *ABCB1* and *CYP2C19* should be considered.

Contributors

JLM and MSS conceived of and designed the research. SDW and EMA acquired the data. JLM, SLC, and MSS analysed and interpreted the data. LS did statistical analyses. JLM and MSS drafted the initial report. JRW and EB participated in funding and supervision. JLM, SLC, SDW, JRW, TS, EMA, EB, and MSS made critical revisions to the report for important intellectual content.

Conflicts of interest

The TIMI Study Group receives research grant support from Daiichi Sankyo, Eli Lilly, Sanofi-Aventis, Bristol-Myers Squibb, AstraZeneca, Schering-Plough/Merck, Johnson & Johnson, and Bayer Healthcare. Additionally, JLM is supported in part by grant K99/R00 HL098461-01 from the National Institutes of Health and reports consulting fees from Sanofi-Aventis, Bristol-Myers Squibb, and AstraZeneca. SDW reports consulting fees from Sanofi-Aventis, Bristol-Myers Squibb, AstraZeneca, ARENA, Medco, and Portola, and lecture fees for CME from Schering-Plough, Daiichi Sankyo, Eli Lilly, Novartis, and AstraZeneca. TS reports research grant support from Servier, Pfizer, Daiichi Sankyo, Eli Lilly, Sanofi-Aventis, AstraZeneca, and Caisse d'Assurance Maladie and consulting fees from Eli Lilly, Daiichi Sankyo, Sanofi-Aventis, Bristol-Myers Squibb, and AstraZeneca. EMA has no additional relationships to disclose. EB reports consulting fees from Daiichi Sankyo and lecture fees from Schering-Plough and Merck. MSS reports consulting fees from AstraZeneca, Bristol-Myers Squibb, Sanofi-Aventis, Daiichi Sankyo, and Eli Lilly and lecture fees from Bristol-Myers Squibb, Sanofi-Aventis, and Eli Lilly. JRW is an employee of Daiichi Sankyo and holds equity ownership or stock options therein. SLC is a former employee of Eli Lilly and holds equity ownership or stock options therein. LS is an employee of Eli Lilly and holds equity ownership or stock options therein.

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Exhibit 39

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**Responding to the Clopidogrel Warning by the US Food and Drug
Administration: Real Life Is Complicated**

Dan M. Roden and Alan R. Shuldiner

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Responding to the Clopidogrel Warning by the US Food and Drug Administration Real Life Is Complicated

Dan M. Roden, MD; Alan R. Shuldiner, MD

A sine qua non for drug approval by the US Food and Drug Administration (FDA) is demonstrated efficacy in populations of patients. However, it is virtually axiomatic that individuals vary in their responses to drugs. Work over decades has built a knowledge base that describes the role of genetic variation as a modulator of both drug efficacy and rare adverse drug effects.¹ This increasing understanding of the role of genetics in variable drug responses led the FDA in 2007 to begin to incorporate pharmacogenetic information in drug labels.²

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In some cases, available data have allowed drug labeling to include specific and highly directive advice. For example, empirical studies, followed by a large randomized clinical trial, demonstrated that preprescription genotyping to avoid the antiretroviral agent abacavir in patients carrying the human leukocyte antigen B*5701 variant can strikingly reduce, if not eliminate, the risk of drug-related severe skin reactions.^{3,4} The FDA label now carries the unambiguous warning stating that such testing should be done and the drug not prescribed in patients with the variant. However, it is likely that single genetic variants with such large effects and predictive value on drug response or adverse effects are more often the exception than the rule; rather, a few or many genetic variants, each with relatively modest effect, contribute to a continuum of drug response in the treated population. Defining the clinical utility of such genetic variants poses important challenges to how pharmacogenetic information may be incorporated into practice. The widespread use of clopidogrel, with its well-documented large interindividual variation in response to the drug and the emerging understanding of the genetics of that variability, is the latest example of such a challenge.

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

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What Is Known

Remarkably, when clopidogrel was approved in 1997, its mechanism of action was not known. Great interindividual variability in response was recognized soon after,⁵ and since then, we have learned that clopidogrel must first be converted to an active metabolite, which then binds and irreversibly inhibits P2Y₁₂ (ADP) receptors on platelets to exert its antiplatelet effect.⁶ Studies indicate that this bioactivation step is largely but not exclusively dependent on the activity of a specific hepatic cytochrome P450 enzyme, termed CYP2C19.⁷ There are several common variants of the *CYP2C19* gene. The normally functioning allele is termed *1, but the *2 allele, which results in loss of function of the encoded protein, is common across many populations. Homozygotes for the loss of function allele (poor metabolizers) represent 2% to 3% of whites and blacks, and up to 15% to 20% of East Asians; heterozygotes represent 30% to 35% and 40% to 45% of these populations, respectively. When ex vivo measures of platelet aggregation are used to define drug effect, loss of function alleles can be shown to decrease drug action in a gene-dose dependent fashion^{7,8}; that is, individuals treated with clopidogrel with the *2/*2 genotype are less responsive than those with the *1/*2 genotype (intermediate metabolizers) who in turn are less responsive than those with the *1/*1 genotype. The surrogate end point of inhibition of platelet aggregation has been partially validated by retrospective examinations of outcomes in patients receiving the drug for clinical indications, in which *2/*2 homozygotes (and possibly also *1/*2 heterozygotes) display increased cardiovascular event rates compared to those with the *1/*1 genotype.⁸⁻¹⁰ These recent findings have led to the FDA-mandated black box label for clopidogrel that now alerts physicians and patients of the role of common *CYP2C19* gene variants in mediating the drug's actions.

What Is Uncertain

Despite the dependency of clopidogrel bioactivation on CYP2C19 activity, not all studies show increased cardiovascular events in patients on clopidogrel with the *1/*2 genotype compared to those with *1/*1.¹⁰ In addition, the effect of rarer *CYP2C19* variants that reduce enzyme function (eg, *3 or *5) has not been studied. Emerging data suggest that *CYP2C19**17, a relatively common allele that results in increased enzyme expression and activity, may be associated with a modest increase in clopidogrel responsiveness.^{11,12} However, the *2 and *17 variants are in linkage disequilibrium, so it is not certain that the effects of this variant are independent of that of the *2 variant.¹³ Some proton pump

inhibitors (PPIs), notably omeprazole,¹⁴ are potent CYP2C19 inhibitors, and omeprazole's reversal of clopidogrel's effect on ex vivo measured platelet function is readily demonstrated. However, there are highly contradictory data on whether coadministration of PPIs and clopidogrel alters cardiovascular event rates.^{15–17}

Most importantly, no studies have been published to define a clinical strategy that would exploit this pharmacogenetic information to optimize outcomes with clopidogrel. Thus, for example, although increasing the dose in $*2/*2$ subjects seems rational, limited available data do not strongly support this strategy.¹⁸ Whereas the FDA's warning does serve to bring the attention of the prescribing community to new data that affects variability in response to drug therapy, the advisory has also generated concern because the practitioner is only offered a series of possible responses, none of which has been tested in any reasonable fashion.

Why Is This So Confusing?

We suggest that one explanation for this confusion arises from differing expectations—in the genetics community, among clinicians, and perhaps among regulators—over the contribution of single genetic variants to common human traits. In the genomics community, there is now an emerging consensus that common gene variants explain a smaller proportion of the heritability of common diseases than had been anticipated.¹⁹ Pharmacogenetics “hype” has promulgated a vision that knowing one or a few genotypes might allow a clear distinction between responders and nonresponders or help identify those likely to suffer catastrophic side effects. This can happen—abacavir is one example—but the reality is that biology is often much more complicated than a few arrows on a simple linear drug response pathway: clopidogrel → bioactivation (by a single gene product) → effect.

In the case of clopidogrel, we do have data: For example, a large study in the Amish, a group with extensive family relationships, showed that the genetic component of variability in the extent to which clopidogrel inhibits ADP-triggered platelet aggregation was $\approx 70\%$.²⁰ A genome-wide association study identified the *CYP2C19* locus as the single most important contributor to this variability, but the contribution of variability was “only” $\approx 12\%$. To a clinician, that may sound like a small number, but to a geneticist this is an enormous contribution. Importantly, there were no other strong association signals apparent in the genome-wide association study suggesting that the majority of the genetic variability in clopidogrel response may be due to more modest effects of many other common variants or perhaps rare or other kinds of genetic variants that escaped detection with current genome-wide association study methodology.

This moderate influence of genetic variation in *CYP2C19* may also explain some of the uncertainties over the PPI effect: it is conceivable that an interaction between PPIs and clopidogrel would only be clinically meaningful in individuals with reduced *CYP2C19* activity (eg, $*1/*2$), whereas $*1/*1$ homozygotes would display sufficient enzyme activity that PPI coadministration would not alter platelet inhibition. This is a hypothesis to be tested, and in any case, as with all drug therapy, it is important to weigh risks and benefits, and

a major benefit of PPIs in this setting is prevention of gastrointestinal hemorrhage.²¹

A recurring theme in complex traits, like pharmacogenomics, is that genetic variation does not confer absolutes, but rather alters probabilities of particular outcomes. This necessarily means that while drug responses may be stochastic (“good” or “bad”) in an individual, this is rarely the case in a population: event rates in patients receiving effective P2Y12 inhibition are not zero, nor are they 100% in patients not receiving drug, or in those genetically unable to generate active drug. Physicians can be quite adept at considering multiple lines of probabilistic evidence-based data in formulating a treatment plan for a given patient. However, they are now presented with an FDA warning on *CYP2C19* and clopidogrel in the face of a gap in knowledge as to how to incorporate the *CYP2C19* genotype into their clinical decision-making practices.

What Response Might a Clinician Adopt?

The accompanying American College of Cardiology Foundation/American Heart Association Clopidogrel Clinical Alert²² nicely outlines possible actions by clinicians:

- Do nothing; follow guidelines: This is a default position, and is tenable in the absence of availability of any other data or testing. This may especially be the case in an interregnum (now) between identification of an important predictor of drug response like *CYP2C19* genotype and solid data on how reasonably to respond to it.
- Use platelet function testing as an alternative to genetic testing: Variability in response to clopidogrel is reminiscent of variable warfarin response; here too, there continues to be argument over the utility of preprescription genotyping as an adjunct to international normalized ratio measurements. The best test of platelet function and how this should be deployed in practice is not yet standardized^{23,24}; One appealing option is to incorporate both genetic testing and platelet function monitoring into management of P2Y12 inhibitor therapy.^{13,24} Initial genetic testing will identify patients at risk for drug failure, whereas intermittent platelet function testing could be viewed as analogous to international normalized ratio measurements for warfarin and allow the clinician to address the variance in drug action even after *CYP2C19* $*2$ is factored in.
- Use preprescription genotyping to guide therapy: Because many cardiovascular events occur within the first few hours to days after percutaneous coronary intervention, a rapid turnaround time is essential. The questions here are how and whether to adjust clopidogrel dose or to choose an alternative drug; and in whom: just poor metabolizers ($*2/*2$ homozygotes) or also in intermediate metabolizers ($*1/*2$ heterozygotes)? In addition, third-party payers may or may not reimburse for genetic testing without the evidence base to support its efficacy.
- Ignore clopidogrel and prescribe “alternate P2Y12 inhibitors” (ie, prasugrel for now) to all: Prasugrel action does not appear to be affected by *CYP2C19* genotype. In the Trial to Assess Improvement in Therapeutic Outcomes by

Optimizing Platelet Inhibition With Prasugrel—Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38) trial, the drug resulted in fewer cardiovascular events but more bleeding.²⁵ Thus, use of prasugrel in all patients would preempt *CYP2C19* genetic testing but increase exposure to adverse bleeding complications. To increase the benefit: risk ratio and manage costs, a more individualized approach might be to prescribe clopidogrel in patients without at-risk genotypes and other drugs such as prasugrel in subjects with *CYP2C19* variant genotypes. This option might also be cost effective, with clopidogrel coming off patent and soon to be much less expensive than newer agents. However, as the American College of Cardiology Foundation/American Heart Association Clopidogrel Clinical Alert correctly points out, the evidence base for this option currently does not exist.

It is clear that none of these options are well supported by data and that major issues are unsettled: eg, which platelet function test is best, how to get timely genetic data on which to act, how to act, and the economics of genetic testing versus complications avoided.

Practice Versus Regulation

The drug label is meant to convey important information for drug use and for marketing.²⁶ Thus, we believe that the FDA has little choice but to inform prescribers of new information that may affect the way in which their patients respond to drugs. To ignore the *CYP2C19* data would be to place the regulatory agency in the unconscionable position of having a label that does not accurately describe the risks and benefits of drug treatment.

The uncertainties over the use of genetic testing in the management of clopidogrel and other drugs, such as warfarin or tamoxifen, reflect impressive progress in pharmacogenetics coupled to uncertainties over how to incorporate that progress into practice. This is the paradox of evidence-based medicine in populations versus individualized medicine. Whereas the “gold standard” for altering practice is the randomized clinical trial, a major challenge remains development of methods to deploy what we know about genomic variation and human traits. The conduct of randomized clinical trials in large unselected populations, most of whom will not carry risk alleles, is inefficient and cost prohibitive. Thus, it will be important to consider novel study designs such as genotype enrichment in populations at high risk for events, and comparative effectiveness study designs incorporating genetics that clearly define treatment options superior to the current standard of care.

Ignoring the newly emerging data on *CYP2C19* genotype and clopidogrel response does not seem to be the best approach. Another way of looking at the tension in this area is to pose the question: “If the genotyping data were readily and simply available at the time of prescribing, should it be used?” Stated this way, the answer would almost certainly be “yes”: there seems little downside to at least knowing which patients can take the standard dose of the about-to-be cheaper drug and which need extra thought. This idea, which might easily apply to many drugs, can be posed because of an

extraordinarily rapidly evolving genotyping environment: We are 1 to 2 years (at most) away from sub-\$1000 whole genome sequencing. This kind of technological development, which raises a myriad of operational, ethical, educational, interpretative, and regulatory challenges,²⁷ will enable a much broader view of how near-future pharmacogenomic discoveries will be translated into clinical practice.

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KEY WORDS: Editorials ■ Food and Drug Administration ■ drug approval ■ individualized medicine ■ pharmacogenetics ■ clopidogrel

Exhibit 40

Coronary Heart Disease

Greater Clinical Benefit of More Intensive Oral Antiplatelet Therapy With Prasugrel in Patients With Diabetes Mellitus in the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel–Thrombolysis in Myocardial Infarction 38

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Background—Patients with diabetes mellitus (DM) are at high risk for recurrent cardiovascular events after acute coronary syndromes, in part because of increased platelet reactivity. The Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel–Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38) showed an overall reduction in ischemic events with more intensive antiplatelet therapy with prasugrel than with clopidogrel but with more bleeding. We compared prasugrel with clopidogrel among subjects with DM in TRITON-TIMI 38.

Methods and Results—We classified 13 608 subjects on the basis of preexisting history of DM and further according to insulin use. Prespecified analyses of the primary (cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke) and key secondary end points, including net clinical benefit (death, nonfatal myocardial infarction, nonfatal stroke, and nonfatal TIMI major bleeding) were compared by use of the log-rank test. We found that 3146 subjects had a preexisting history of DM, including 776 receiving insulin. The primary end point was reduced significantly with prasugrel among subjects without DM (9.2% versus 10.6%; hazard ratio [HR], 0.86; $P=0.02$) and with DM (12.2% versus 17.0%; HR, 0.70; $P<0.001$, $P_{\text{interaction}}=0.09$). A benefit for prasugrel was observed among DM subjects on insulin (14.3% versus 22.2%; HR, 0.63; $P=0.009$) and those not on insulin (11.5% versus 15.3%; HR, 0.74; $P=0.009$). Myocardial infarction was reduced with prasugrel by 18% among subjects without DM (7.2% versus 8.7%; HR, 0.82; $P=0.006$) and by 40% among subjects with DM (8.2% versus 13.2%; HR, 0.60; $P<0.001$, $P_{\text{interaction}}=0.02$). Although TIMI major hemorrhage was increased among subjects without DM on prasugrel (1.6% versus 2.4%; HR, 1.43; $P=0.02$), the rates were similar among subjects with DM for clopidogrel and prasugrel (2.6% versus 2.5%; HR, 1.06; $P=0.81$, $P_{\text{interaction}}=0.29$). Net clinical benefit with prasugrel was greater for subjects with DM (14.6% versus 19.2%; HR, 0.74; $P=0.001$) than for subjects without DM (11.5% versus 12.3%; HR, 0.92; $P=0.16$, $P_{\text{interaction}}=0.05$).

Conclusions—Subjects with DM tended to have a greater reduction in ischemic events without an observed increase in TIMI major bleeding and therefore a greater net treatment benefit with prasugrel compared with clopidogrel. These data demonstrate that the more intensive oral antiplatelet therapy provided with prasugrel is of particular benefit to patients with DM. (*Circulation*. 2008;118:1626-1636.)

Key Words: angioplasty ■ anticoagulants ■ myocardial infarction ■ platelets ■ diabetes mellitus

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The global prevalence of diabetes mellitus (DM) has been estimated at >170 million individuals and is rapidly increasing, with projections of >350 million by 2030.¹ With improved control over other cardiovascular risk factors, the burden of cardiovascular disease attributable to DM is increasing.^{2,3} Among patients with acute coronary syndromes (ACS), those with DM are at higher risk for subsequent events, including death, independent of other comorbidities⁴; indeed, patients with DM but without known cardiovascular disease are at a risk of morbidity and mortality after ACS similar to that of patients without DM but with known cardiovascular disease.⁵ Several mechanisms may play roles in the increased risk of events in patients with DM, including greater frequency of other cardiac risk factors, a greater burden of atherosclerotic disease, hyperglycemia, inflammation, and a greater tendency toward thrombosis.^{6–8} Despite initial favorable data, results of definitive studies of insulin therapy in patients with ACS have not demonstrated benefit.^{9–11}

Platelet activation and aggregation are key factors in the development of ACS and its complications. It has been known for decades that platelets from patients with DM are characterized by increased reactivity.^{12,13} Intensive intravenous blockade of platelet aggregation in subjects with ACS using glycoprotein IIb/IIIa receptor inhibitors (GPIs) appears to be of particular benefit among subjects with DM.^{7,14} A meta-analysis of >30 000 subjects from the acute GPI trials revealed a significant 26% relative reduction in mortality among subjects with DM, a significant 70% relative mortality reduction among diabetic subjects undergoing percutaneous coronary intervention (PCI), and no mortality benefit among subjects without DM.^{7,14}

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Prasugrel is a third-generation thienopyridine antiplatelet agent that, like clopidogrel, exerts its antiplatelet effect by P2Y₁₂ receptor blockade.¹⁵ Treatment with prasugrel results in higher and more consistent levels of platelet inhibition than standard- or higher-dose clopidogrel.^{16,17} In the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel–Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38), treatment with prasugrel compared with clopidogrel resulted in a 19% lower incidence in cardiovascular death, nonfatal myocardial infarction (MI), or nonfatal stroke but with more bleeding among patients with ACS in whom PCI was planned.¹⁸ Several studies using multiple methods of measurement of platelet function have demonstrated less inhibition and greater rates of poor antiplatelet response to clopidogrel among subjects with DM.^{19–23} We hypothesized that because of the increased platelet reactivity and higher reported rates of poor response to clopidogrel among patients with DM, we would observe a greater benefit for prasugrel compared with clopidogrel among subjects with DM in TRITON-TIMI 38.

Methods

Study Population

This analysis includes all 13 608 subjects randomized into the TRITON-TIMI 38 trial stratified by DM status, a prespecified

subgroup. Inclusion and exclusion criteria for the main trial have previously been published in detail.²⁴ Briefly, subjects were eligible for enrollment if they had moderate- to high-risk unstable angina (UA/NSTEMI), after medical treatment for ST-segment elevation MI (STEMI) with coronary anatomy known to be suitable for PCI, or before cardiac catheterization with planned primary PCI for STEMI. Key exclusion criteria included increased risk of bleeding and any thienopyridine within 5 days before enrollment. A history of DM for each subject was established from the report of the local investigator on the case record form. Subjects were classified on the basis of treatment with insulin before enrollment in TRITON-TIMI 38.

End Points

End-point definitions for TRITON-TIMI 38 have been published previously and were used for this analysis.^{18,24} All components of the end points were adjudicated by a clinical events committee blinded to treatment assignment. The primary end point of TRITON-TIMI 38 and this analysis was the composite of cardiovascular death, nonfatal MI, or nonfatal stroke. Additional efficacy end points examined included the individual assessment of cardiovascular death, MI, or stent thrombosis using the definite/probable Academic Research Consortium definition.^{25,26} The key safety end point was non-coronary artery bypass grafting (CABG)-related TIMI major bleeding; secondary analysis was of non-CABG-related TIMI major or minor bleeding as previously defined.²⁴ Net clinical benefit was defined as the composite of all-cause mortality, nonfatal MI, nonfatal stroke, or nonfatal TIMI major bleeding not related to CABG.¹⁸ Efficacy event rates are calculated from intention-to-treat analyses, and safety analyses are based on the safety cohort.¹⁸

Statistical Analysis

Baseline characteristics of subjects with and without DM were compared by the χ^2 test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Because randomization was not stratified by DM status, baseline characteristics were compared among subjects with and without DM by treatment assignment. Survival analysis methods were used to compare outcomes by treatment assignment (prasugrel versus clopidogrel) and to compare outcomes by presence or absence of DM. Event rates are reported using Kaplan–Meier estimates at 450 days. Comparisons are expressed as hazard ratios (HRs) and 95% CIs including the entire duration of follow-up. Testing for an interaction between the efficacy of prasugrel compared with clopidogrel and diabetic status was performed by constructing a Cox proportional-hazards model using terms for both the main effect and the interaction. Given the number of subjects enrolled, the proportion of subjects with DM, and the overall reduction in the primary end point with prasugrel in the total cohort, there was 80% power at an α level of 0.05 to detect an interaction HR (ie, the ratio of the HRs for benefit of prasugrel in the DM and non-DM groups) of 0.70; assuming an $\alpha=0.10$, there was 80% power to detect an interaction HR of 0.73. For all analyses, values of $P<0.05$ were considered significant. All analyses were performed with STATA/SE 9.2 (STATA Corp, College Station, Tex).

The sponsors of TRITON-TIMI 38 supported the design and implementation of the main trial from which these results are obtained. All analyses were performed by the TIMI Study Group using an independent copy of the complete clinical trial database. The authors wrote all drafts of the article and take responsibility for its content. The sponsors had the opportunity to review and comment on this article but had no editorial authority.

Results

Baseline and Procedural Characteristics

Of 13 608 subjects randomized into TRITON-TIMI 38, 3146 (23%) had DM, with 776 (6%) reporting treatment with insulin. Baseline characteristics by diabetic status are shown in Table 1. Subjects with DM were more likely to have UA/NSTEMI, to be older, to more often be female, and to

Table 1. Baseline Characteristics

Characteristic	Subjects With DM (n=3146)	Subjects Without DM (n=10 462)	P
UA/NSTEMI, %	79	73	<0.001
STEMI, %	21	27	
Age, median (25th, 75th percentiles), %	63 (55, 71)	60 (52, 69)	<0.001
Age ≥ 75 y, %	15	13	<0.001
Female gender, %	33*	24	<0.001
BMI, median (25th, 75th percentiles), %	29 (26, 33)	27 (25, 30)	<0.001
Weight, %			<0.001
<70 kg	15	20	
70–90 kg	45	52	
>90 kg	40	29	
White race, %	89	94	<0.001
Region of enrollment, %			<0.001
North America	36	31	
Western Europe	22	27	
Eastern Europe	21	25	
Middle East/Africa/Asia Pacific	16	13	
South America	5	4	
History of hypertension, %	80	59	<0.001
History of hypercholesterolemia, %	67	52	<0.001
Current tobacco use, %	27	41	<0.001
Prior MI, %	23	16	<0.001
Prior CABG, %	12	6*	<0.001
Prior CVA/TIA, %	6	3	<0.001
Creatinine clearance <60 mL/min, %	14	10	<0.001
Bare metal stent only, %	43	50	
Drug-eluting stent only, %	52	46	<0.001
Multivessel PCI, %	17	13	<0.001
GPI use, %†	53	55	0.06
Pharmacotherapies, %			
ACE/ARB	85	73	<0.001
β -Blocker	89	88	0.16
Statin	92	92	0.37
CCB	24	16	<0.001
ASA	99	99	0.81

BMI indicates body mass index; CVA, cerebrovascular accident; TIA, transient ischemic attack; ACE, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker, and ASA, aspirin. Probability value is for the comparison of subjects with DM vs subjects without DM.

* $P < 0.05$ between subjects assigned to prasugrel vs clopidogrel within diabetes stratum (see text).

†Use during index hospitalization.

have a higher body mass index. DM subjects were more often from North America, had more frequent prior MI and prior CABG, and were more likely to have hypertension or hyperlipidemia but were less likely to be smokers. There was no difference in the frequency of PCI at the time of enroll-

ment. For the index PCI, subjects with DM were more likely to have multivessel PCI and were more likely to have received at least 1 drug-eluting stent than subjects without DM. Subjects with DM were more likely to receive treatment with an angiotensin-converting enzyme inhibitor, an angiotensin receptor blocker, or a calcium channel blocker but had similar rates of use of GPIs, statins, and aspirin. Baseline characteristics were well matched within the randomized treatment assignments (prasugrel versus clopidogrel). The only significant differences between treatment groups were that among subjects with DM more women were randomized to clopidogrel than prasugrel (36% versus 31%; $P = 0.004$) and that among nondiabetics slightly more subjects randomized to prasugrel had prior CABG (7% versus 6%; $P = 0.03$).

Compared with subjects with DM not on insulin (Table 1 of the online Data Supplement), DM subjects on insulin were slightly younger, more often female, heavier, and more often enrolled in North America and more often had prior MI, prior CABG, prior stroke, and renal dysfunction. Subjects on insulin were less likely to receive only bare metal stents but otherwise had similar adjunctive therapies.

Outcomes by Diabetic Status

Rates of thrombotic events were higher among subjects with DM than subjects without DM (Table 2). The primary end point was observed in 14.6% of subjects with and 9.9% of subjects without DM (HR, 1.45; 95% CI, 1.29 to 1.62; $P < 0.0001$). A significantly increasing gradient of higher event rates was observed with a progression across cohorts from no DM to DM without insulin to DM on insulin (Table 3). The primary end point was seen in 9.9% of subjects without DM, 13.4% of subjects with DM without insulin therapy, and 18.3% of subjects with insulin therapy (P for trend < 0.0001). MI was observed in 8.0% of subjects without DM and 10.7% of subjects with DM (HR, 1.30; 95% CI, 1.14 to 1.49; $P = 0.0001$), with a similar gradient observed in the 3-stratum comparison (Table 3). Subjects with DM had a higher rate of stent thrombosis than those without DM (2.8% versus 1.4%; HR, 1.95; 95% CI, 1.47 to 2.59; $P < 0.0001$), with highest rates among subjects treated with insulin. After baseline demographic and treatment differences were controlled for, DM remained an independent predictor of ischemic outcomes, including the primary end point, MI, stent thrombosis, and net clinical benefit.

Overall, major bleeding rates (2.6% versus 2.0%; HR, 1.28; $P = 0.08$) and major or minor bleeding rates (4.8% versus 4.2%; HR, 1.15; 95% CI, 0.95 to 1.4; $P = 0.15$) were similar between subjects with and without DM, respectively, regardless of diabetes treatment.

Prasugrel Compared With Clopidogrel by Diabetes Status

A significant 14% overall reduction in the primary end point (Table 4 and Figure 1A) was seen with prasugrel among subjects without DM ($P = 0.02$). Among subjects with DM, a 30% reduction in the primary end point was observed with prasugrel ($P < 0.001$, $P_{\text{interaction}} = 0.09$). As a result of the relative benefits and event rates, the number needed to treat with prasugrel to prevent 1 primary end-point event among

Table 2. Clinical Events by Diabetes Status (DM Versus No DM)

	No DM (n=10 462), %	DM (n=3146), %	HR (95% CI)	P
CVD/MI/CVA*	9.9	14.6	1.45 (1.29–1.62)	<0.0001
CVD/MI	9.2	13.1	1.39 (1.23–1.56)	<0.0001
MI†	8.0	10.7	1.30 (1.14–1.49)	0.0001
CVD	1.8	3.8	2.13 (1.68–2.70)	<0.0001
Stent thrombosis	1.4	2.8	1.95 (1.47–2.59)	<0.0001
Major bleed‡	2.0	2.6	1.28 (0.97–1.68)	0.08
Major or minor bleed‡	4.2	4.8	1.15 (0.95–1.41)	0.15
D/MI/CVA*/major bleed‡	11.9	16.9	1.40 (1.26–1.56)	<0.0001

CVD indicates cardiovascular death; CVA, cerebrovascular accident; and D, death.

*The composite of cardiovascular death, nonfatal MI, or nonfatal stroke.

†Any MI (fatal or nonfatal).

‡Not related to CABG.

subjects with DM was 21 compared with 71 subjects without DM. The reduction in the primary end point among subjects with DM was consistent across major subgroups (Figure 2). There were no significant interactions between treatment effect among patients with DM and presenting syndrome (UA/NSTEMI versus STEMI), gender, age, use of GPI, renal function, or stent type. The reduction in the primary end point with prasugrel was driven largely by a lower incidence of MI (Figure 1B). A highly significant 18% reduction in MI among subjects without DM ($P=0.006$) was observed, and there was a statistically greater 40% reduction in MI in subjects with DM ($P<0.001$, $P_{\text{interaction}}=0.02$). The substantial reduction in stent thrombosis (Figure 1C) with prasugrel was similar regardless of diabetic status, including 55% among those without DM (2.0% versus 0.9%; $P<0.001$) and 48% among subjects with DM (3.6% versus 2.0%; $P=0.007$, $P_{\text{interaction}}=0.63$). Among patients with DM, stent thrombosis was reduced to a similar extent with prasugrel in the 1605 patients treated with a drug-eluting stent only (2.0% with prasugrel versus 3.5% with clopidogrel; HR, 0.53; 95% CI, 0.28 to 1.02; $P=0.054$) and the 1327 patients with bare metal stents only (1.9% versus 3.8%; HR, 0.52; 95% CI, 0.26 to 1.04; $P=0.06$, $P_{\text{interaction}}=0.95$).

Among subjects without DM, prasugrel treatment was associated with a 43% increase in non-CABG-related TIMI

major hemorrhage ($P=0.02$). Although no significant difference was seen in major hemorrhage in those with DM, no interaction between treatment and DM status was observed for major hemorrhage (Figure 1D). Among patients without DM, TIMI major or minor bleeding not related to CABG was observed in 4.9% with prasugrel and 3.6% with clopidogrel (HR, 1.32; 95% CI, 1.08 to 1.61; $P=0.006$). Among patients with DM, TIMI major or minor bleeding was observed in 5.3% with prasugrel and 4.3% with clopidogrel (HR, 1.30; 95% CI, 0.92 to 1.82; $P=0.13$, $P_{\text{interaction}}=0.93$).

The combination of a relatively greater reduction in ischemic end points and no increase in major bleeding among subjects with DM led to a statistically greater net clinical benefit for prasugrel among subjects with DM (Figure 1E). Specifically, among subjects without DM, a nonsignificant 8% reduction in the composite of all-cause mortality, nonfatal MI, nonfatal stroke, or nonfatal major bleeding ($P=0.16$) was observed, whereas a statistically greater 26% reduction in this composite outcome was seen for subjects with DM ($P=0.001$, $P_{\text{interaction}}=0.05$).

Prasugrel Compared With Clopidogrel by Diabetes Treatment Type

The relative benefit of prasugrel compared with clopidogrel for the reduction of ischemic end points was consistent across

Table 3. Clinical Events by Diabetes Status (No DM, DM but No Insulin, DM With Insulin)

	No DM (n=10 462), %	DM, No Insulin (n=2370), %	HR (95% CI) vs No DM	DM With Insulin (n=776), %	HR (95% CI) vs No DM	P for Trend*
CVD/MI/CVA*	9.9	13.4	1.32 (1.16–1.51)	18.3	1.84 (1.53–2.20)	<0.0001
CV death/MI	9.2	12.0	1.27 (1.11–1.46)	16.3	1.74 (1.44–2.11)	<0.0001
MI†	8.0	9.8	1.18 (1.01–1.37)	13.7	1.69 (1.37–2.08)	<0.0001
CV death	1.8	3.5	2.02 (1.55–2.64)	4.7	2.46 (1.68–3.61)	<0.0001
Stent thrombosis	1.4	2.5	1.73 (1.25–2.39)	3.7	2.65 (1.73–4.06)	<0.0001
Major bleed‡	2.0	2.7	1.33 (0.99–1.80)	2.1	1.12 (0.66–1.89)	0.16
Major or minor bleed‡	4.2	4.9	1.18 (0.95–1.46)	4.4	1.08 (0.75–1.55)	0.24
D/MI/CVA*/major bleed‡	11.9	15.8	1.31 (1.16–1.47)	20.5	1.71 (1.44–2.03)	<0.0001

Abbreviations as in Table 2.

*The composite of cardiovascular death, nonfatal MI, or nonfatal stroke.

†Any MI (fatal or nonfatal).

‡Not related to CABG.

Table 4. Clinical Events for Prasugrel Versus Clopidogrel by Diabetes Status

	Clopidogrel, %	Prasugrel, %	HR (95% CI)	P	P _{interaction} vs No Diabetes
Subjects without DM (n=10 462), n	5225	5237			
CVD/MI/CVA*	10.6	9.2	0.86 (0.76–0.98)	0.02	
CVD/MI*	10.0	8.5	0.85 (0.75–0.97)	0.01	
MI†	8.7	7.2	0.82 (0.72–0.95)	0.006	
CV death	1.9	1.7	0.91 (0.68–1.23)	0.53	
Stent thrombosis	2.0	0.9	0.45 (0.31–0.65)	<0.001	
Major hemorrhage‡	1.6	2.4	1.43 (1.07–1.91)	0.02	
Major or minor‡	3.6	4.9	1.32 (1.08–1.61)	0.006	
D/MI/CVA*/major bleed‡	12.3	11.5	0.92 (0.82–1.03)	0.16	
All diabetes (n=3148), n	1570	1576			
CVD/MI/CVA*	17.0	12.2	0.70 (0.58–0.85)	<0.001	0.09
CVD/MI*	15.4	10.8	0.68 (0.56–0.84)	<0.001	0.08
MI†	13.2	8.2	0.60 (0.48–0.76)	<0.001	0.02
CV death	4.2	3.4	0.85 (0.58–1.24)	0.40	0.78
Stent thrombosis	3.6	2.0	0.52 (0.33–0.84)	0.007	0.63
Major hemorrhage‡	2.6	2.5	1.06 (0.66–1.69)	0.81	0.29
Major or minor‡	4.3	5.3	1.30 (0.92–1.82)	0.13	0.93
D/MI/CVA*/major bleed‡	19.2	14.6	0.74 (0.62–0.89)	0.001	0.05

Abbreviations as in Table 2.

*The composite of cardiovascular death and nonfatal end points (MI alone or MI/stroke).

†Any MI (fatal or nonfatal).

‡Not related to CABG.

the different treatment subgroups of diabetes (insulin versus no insulin; Table 5 and Figure 3). Among insulin-treated and non-insulin-treated diabetics, highly significant relative reductions in the primary end point (37% and 26%, respectively) were observed. This resulted in a number needed to treat of 13 subjects with DM on insulin and 26 with DM not on insulin to prevent 1 primary end-point event. Substantial benefits in ischemic events, including a 44% relative reduction in MI (9.9% versus 17.3%; $P=0.005$) for DM on insulin and a 38% relative reduction for MI in DM without insulin (11.9% versus 7.7%; $P<0.001$), plus a 69% relative reduction in stent thrombosis for DM on insulin (1.8% versus 5.7%; $P=0.008$) and a 34% reduction among subjects with DM without insulin therapy (2.0% versus 3.0%; $P=0.14$), were observed. Hemorrhage rates were similar regardless of DM treatment type. As a result, a net clinical benefit of 34% was seen among subjects with DM on insulin ($P=0.01$) and a 22% net clinical benefit among subjects with DM not on insulin ($P=0.02$).

Discussion

We demonstrated in TRITON-TIMI 38 that more intensive and consistent antiplatelet therapy with a third-generation thienopyridine (prasugrel) plus aspirin in subjects with ACS undergoing PCI resulted in a reduction in ischemic events compared with standard dual antiplatelet therapy but with more bleeding and, on balance, an improved net clinical benefit.¹⁸ The present analysis examined the effect of DM on outcomes and the relative efficacy of prasugrel compared with clopidogrel. Our data extend previous observations regarding worse clinical outcomes of subjects with DM

across the spectrum of ACS.⁴ We observed that despite modern coronary intervention with stenting and high levels of guideline-based medical care, DM had an independent adverse effect on clinical outcome. In addition, we observed that subjects with DM treated with insulin at the time of enrollment had very high rates of ischemic events.

Prasugrel was especially efficacious in patients with DM. Although key ischemic end points, including the primary end point and MI, were significantly reduced among subjects both with and without DM, a greater relative reduction was seen in favor of prasugrel among subjects with DM, including a 40% relative reduction in MI. In addition, when both ischemic events and bleeding are integrated into a net clinical benefit composite end point, a significantly greater relative improvement was observed with prasugrel in subjects with DM than without DM (26% versus 8%). This combination of higher clinical event rates and greater relative treatment effect in subjects with DM led to markedly greater absolute event reductions in subjects with DM. Although these results were consistent regardless of whether subjects with DM were treated with insulin, the higher event rates for subjects with DM on insulin result in an even greater absolute benefit. Compared with standard clopidogrel, to prevent 1 cardiovascular death, nonfatal MI, or nonfatal stroke, 13 patients with DM on insulin, 26 patients with DM not on insulin, and 71 patients without DM would need to be treated with prasugrel.

The present data also build on previous observations regarding intensive antiplatelet therapy among subjects with DM.²⁷ The particular benefit of GPIs among patients with DM has led to the support in clinical guidelines for their use in patients with DM undergoing PCI.²⁸ Speculation regarding

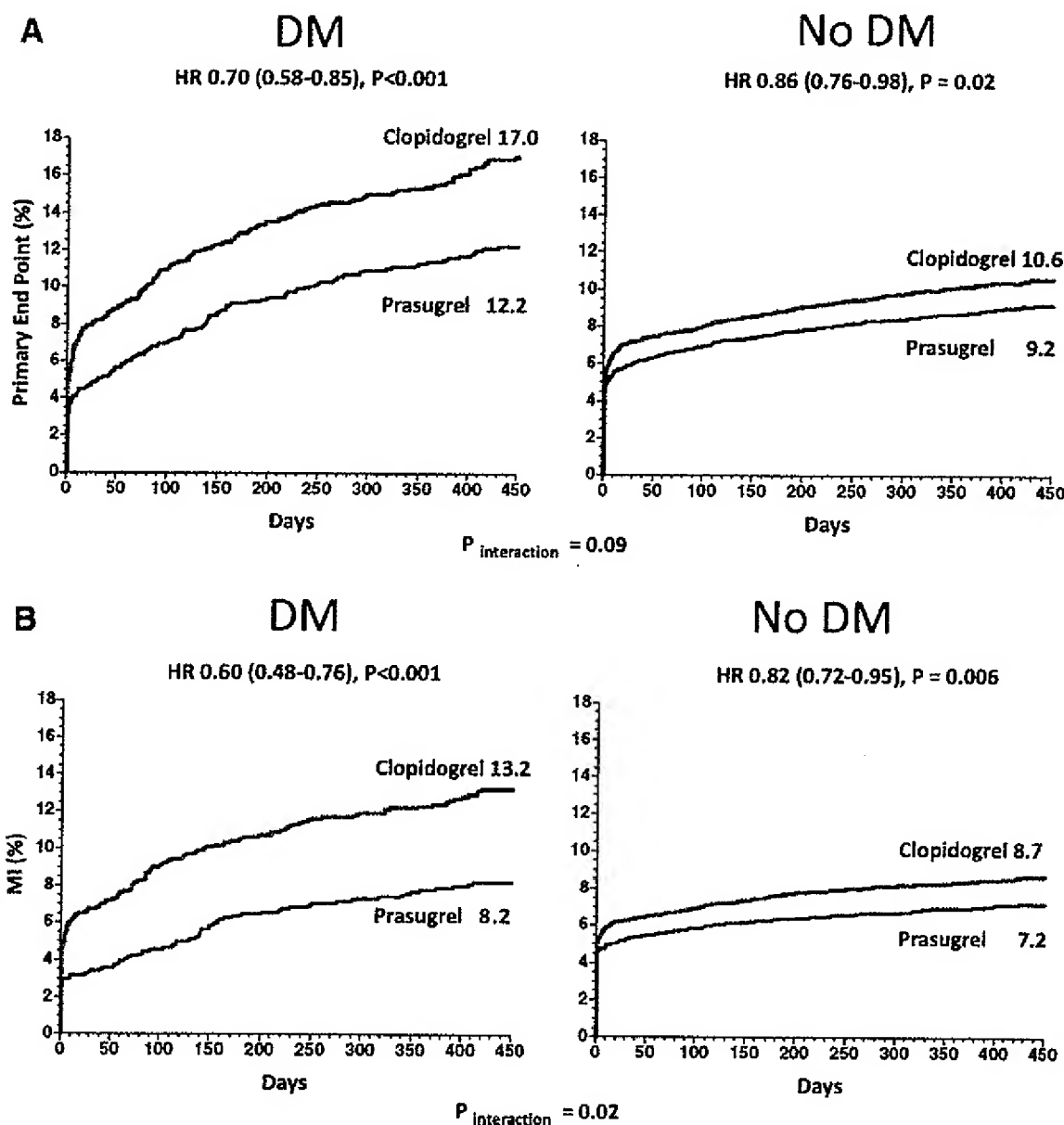


Figure 1. Kaplan-Meier curves for prasugrel vs clopidogrel stratified by diabetes status. A, Primary efficacy end point (cardiovascular death/nonfatal MI/nonfatal stroke) stratified by diabetic status. B, MI (fatal or nonfatal). C, Definite or probable stent thrombosis. D, TIMI major bleeding not related to CABG. E, Net benefit end point (death/nonfatal MI, nonfatal cerebrovascular accident, nonfatal TIMI major bleed not related to CABG).

the mechanisms of the apparent increased benefit of antiplatelet therapy has centered on increased platelet reactivity with DM.^{6,7,29} Several studies have identified differences in platelet reactivity among subjects with and without DM, with greater response to platelet agonists among the former.^{6,12,13,19,22,30,31} Potential mechanisms to explain this difference in platelet activity include direct platelet effects such as glycosylation of platelet membrane proteins, leading to alterations in receptor function and signaling pathways, and nonplatelet effects such as increased oxidative stress and impaired endothelial function, resulting in a procoagulant milieu.⁶ Physiologically, insulin reduces platelet aggregation

by inhibiting the $P2Y_{12}$ receptor, although this inhibition is absent in subjects with DM and insulin resistance.^{32,33} In previous mechanistic studies, insulin-treated subjects had higher levels of platelet aggregation after dual antiplatelet therapy than subjects with DM not treated with insulin, indicating that treatment with insulin may identify a group of subjects at particular risk for poor response to clopidogrel.²² We did not collect age of onset (juvenile versus adult) or duration of diabetes, both factors that may influence insulin use. These data do not necessarily imply that insulin use was causally related to higher rates of adverse outcomes but are compatible with the notion that insulin use identifies a high-risk subgroup.

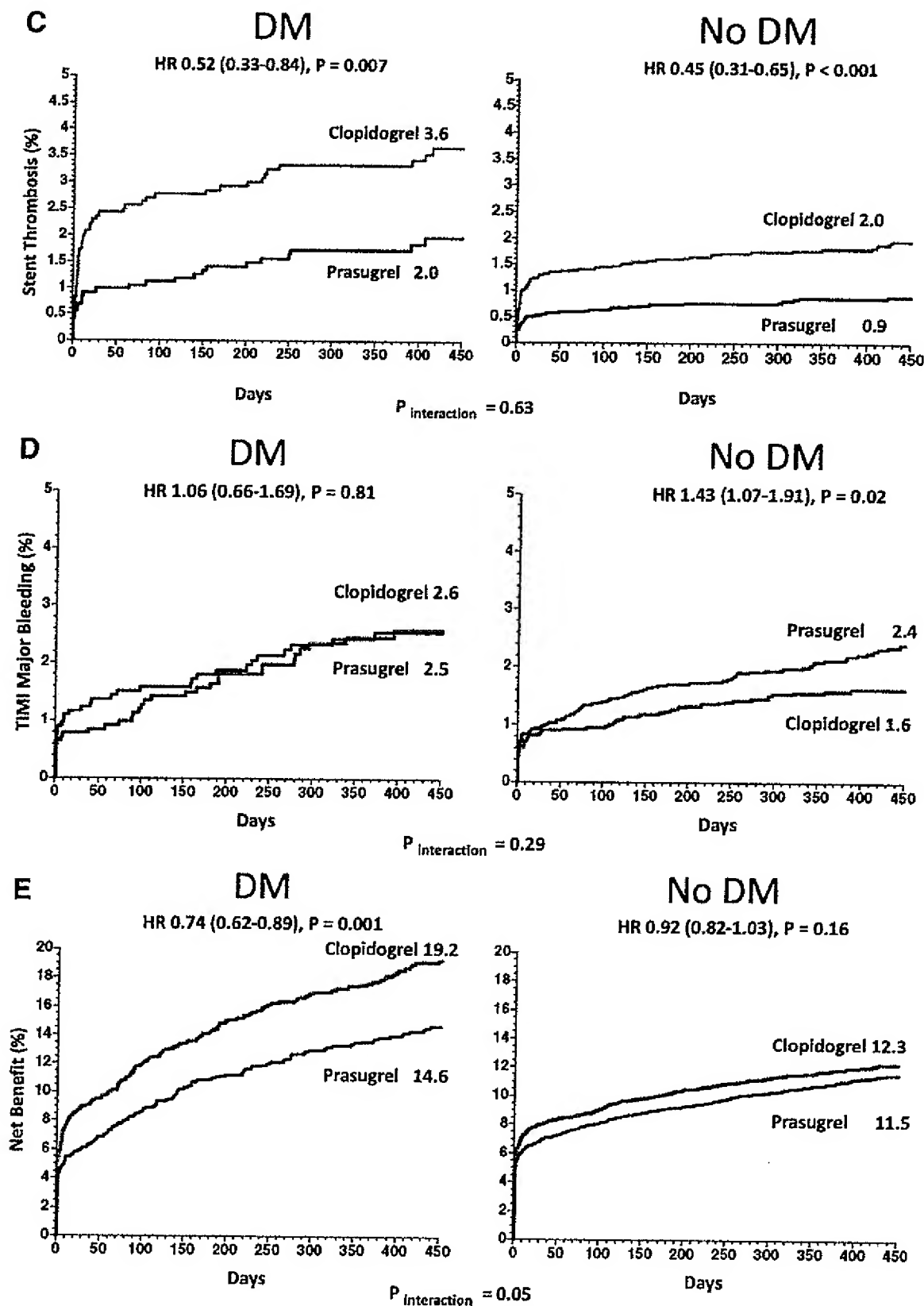


Figure 1 (Continued).

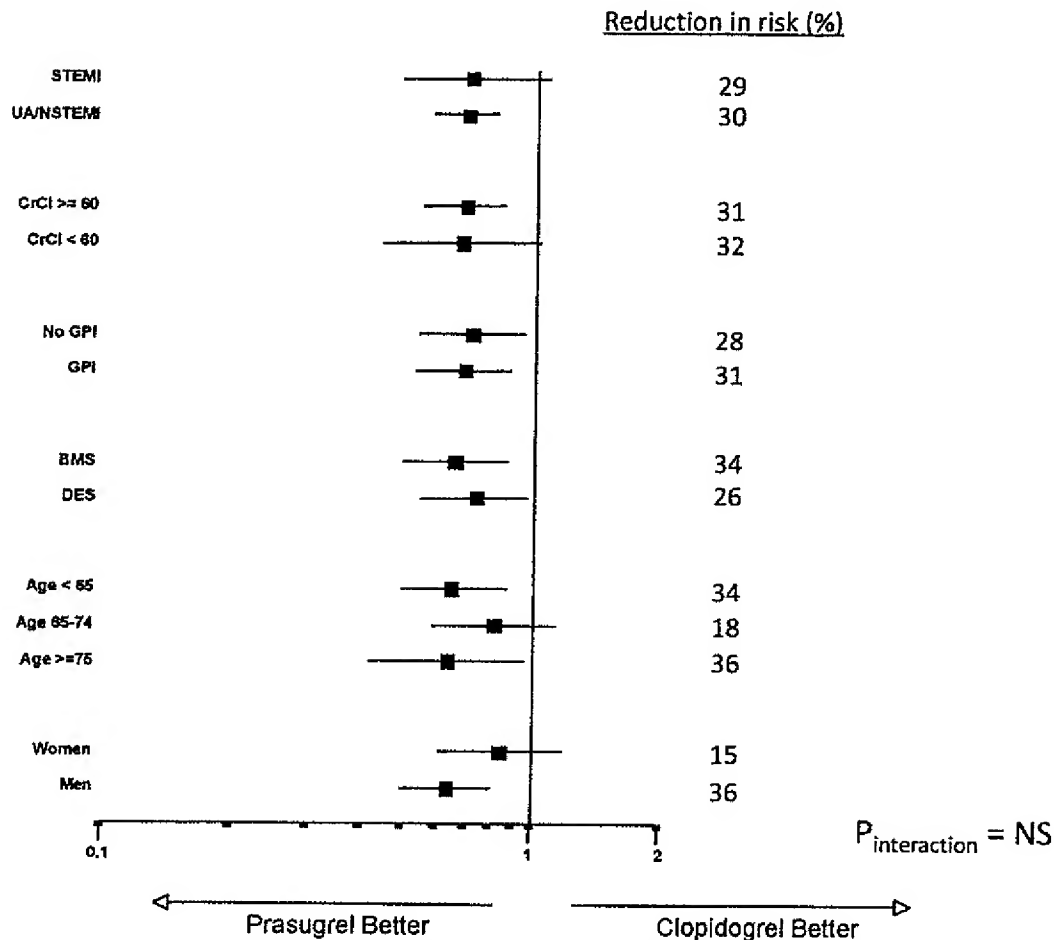


Figure 2. Subgroups among subjects with DM. BMS indicates bare metal stents only; DES, at least 1 drug-eluting stent; GPI, GPIs used during index hospitalization; and CrCl, creatinine clearance.

Given the potential role of stimulation of the $P2Y_{12}$ receptor in platelet activation seen in subjects with DM, a particular benefit of thienopyridines seen in this subgroup is biologically plausible. Indeed, in the Clopidogrel Versus Aspirin in Patients at Risk for Ischemic Events (CAPRIE) trial comparing aspirin with clopidogrel in the secondary prevention of vascular disease events, a greater benefit of clopidogrel among subjects with DM was noted.³⁴ However, in the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial of subjects with ACS, dual antiplatelet therapy with aspirin and clopidogrel was associated with a similar benefit regardless of DM, although event rates were higher among subjects with DM.³⁵

One factor that may have limited the ability to observe a greater effect among subjects with DM and ACS in CURE is hyporesponsiveness to clopidogrel. Intersubject variability of response to clopidogrel is a well-described laboratory phenomenon, with growing evidence for poor response to clopidogrel being associated with worse clinical outcomes.^{17,36,37} Clinical factors associated with impaired response to clopidogrel include both ACS and DM, placing the subjects with these 2 features at especially high risk for a diminished response to clopidogrel.³⁷ Clopidogrel responsiveness has

been specifically tested in subjects with DM and has been observed to be related to clinical outcomes. Diabetic subjects with higher posttreatment platelet reactivity are at higher risk for adverse events after coronary stenting.³⁸ As a result of these observations, investigators have examined the effects of higher (150-mg) maintenance²⁰ doses of clopidogrel on platelet function in subjects with DM and found improved response, but they also noted the persistence of a high proportion of subjects not achieving predefined goals for platelet inhibition.²⁰ The results observed in this DM subgroup analysis of TRITON-TIMI 38, however, are the first demonstration in a trial adequately sized for clinical outcomes that more intensive oral antiplatelet therapy (in this case, with prasugrel) improved clinical outcomes compared with standard-dose clopidogrel in diabetic subjects. Although the data presented here do not address the question of whether prasugrel would have been superior to higher-dose clopidogrel, prasugrel at the doses used in TRITON-TIMI 38 has been shown to provide superior antiplatelet effects to higher loading (600 mg) and maintenance (150 mg) doses of clopidogrel in patients undergoing elective PCI in the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation--Thrombolysis in Myocardial

Table 5. Clinical Events for Prasugrel Versus Clopidogrel by Diabetes Subtype

Event	Clopidogrel, %	Prasugrel, %	HR (95% CI)	P
Subjects with DM on insulin (n=776), n	397	379		
CVD/MI/CVA*	22.2	14.3	0.63 (0.44–0.89)	0.009
CVD/MI*	19.3	13.1	0.64 (0.44–0.93)	0.02
MI†	17.3	9.9	0.56 (0.37–0.84)	0.005
Stent thrombosis	5.7	1.8	0.31 (0.12–0.77)	0.008
Major hemorrhage‡	2.3	1.9	0.87 (0.31–2.39)	0.78
Major or minor‡	4.5	4.4	0.93 (0.46–1.88)	0.84
D/MI/CVA*/major bleed‡	24.1	16.8	0.66 (0.47–0.92)	0.01
DM not on insulin (n=2370), n	1173	1197		
CVD/MI/CVA*	15.3	11.5	0.74 (0.59–0.93)	0.009
CVD/MI*	14.0	10.1	0.70 (0.55–0.89)	0.004
MI†	11.9	7.7	0.62 (0.47–0.82)	<0.001
Stent thrombosis	3.0	2.0	0.66 (0.37–1.15)	0.14
Major hemorrhage‡	2.7	2.7	1.11 (0.65–1.89)	0.70
Major or minor‡	4.2	5.6	1.42 (0.96–2.10)	0.08
D/MI/CVA*/major bleed‡	17.7	13.9	0.78 (0.63–0.96)	0.02

Abbreviations as in Table 2.

*The composite of cardiovascular death and nonfatal end points (MI alone or MI/stroke).

†Any MI (fatal or nonfatal).

‡Not related to CABG.

Infarction 44 trial (PRINCIPLE-TIMI 44) study.¹⁷ The Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent Events–Optimal Antiplatelet Strategy for Interventions (CURRENT-OASIS 7) trial (NCT00335452) is comparing the clinical efficacy of high- and standard-dose clopidogrel; the results of the diabetic patients in this trial will be of great interest in determining whether a smaller difference in the intensity of platelet inhibition will result in improved clinical outcomes similar to those observed in TRITON-TIMI 38.

The greater relative effect of prasugrel compared with clopidogrel among subjects with DM that we observed in this analysis supports the hypothesis that greater platelet inhibition among patients with DM results in improved outcomes. Whether the differential effects between prasugrel and clopidogrel result from a greater level of inhibition of platelet aggregation on a population level or a greater proportion of subjects reaching a specific threshold cannot be determined from these data. That the benefit was consistent among

subjects with DM across a broad spectrum of characteristics, including UA/NSTEMI or STEMI, gender, and age, suggests that there is a relationship between DM per se and greater relative benefit with prasugrel. That a greater absolute benefit is seen among insulin-treated subjects with DM also supports this hypothesis. Of note, in the presence or absence of GPII, the benefit of prasugrel compared with clopidogrel was still observed, indicating that blocking the P2Y₁₂ receptor in patients with DM is an important target beyond potent inhibition of platelet aggregation.

It is of interest that subjects with DM had similar TIMI major bleeding rates regardless of treatment with prasugrel or clopidogrel. This may relate to the higher body weight of diabetics or greater baseline platelet reactivity among diabetics, or because the probability value for interaction with the entire cohort is nonsignificant, this observation may simply have been the play of chance. The latter explanation is supported by the similar relative increase in the combination of major or minor bleeding among subjects with and without DM and the higher major bleeding rate among diabetics compared with nondiabetics on clopidogrel, a finding that would be unexpected if this difference were related only to platelet activity.

The combination of higher ischemic event rates with greater reductions with prasugrel and similar major bleeding rates leads to a significantly greater net benefit of prasugrel compared with clopidogrel among subjects with DM. In choosing antiplatelet therapies for management of patients with ACS undergoing PCI, the treating physician needs to weigh the competing risks of recurrent thrombosis and bleeding. Previously, subgroups have been identified that had limited net benefit with prasugrel from TRITON-TIMI 38, including those with prior stroke or low body weight or the

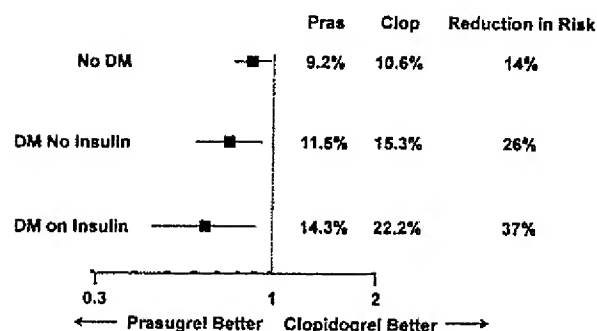


Figure 3. Reduction in the primary end point (cardiovascular death/nonfatal MI, nonfatal stroke) by diabetes status and treatment group. Pras indicates prasugrel; clop, clopidogrel.

elderly.¹⁸ These data demonstrate that patients with DM, seen in increasing frequency with ACS, derive a particular benefit from a more intensive oral antiplatelet strategy with acceptable safety.

Study Limitations

This was a prespecified subgroup analysis of prasugrel versus clopidogrel among diabetics. Information regarding diabetes type was posthoc and should be considered exploratory. Comparisons between the outcomes of subjects by diabetic status (diabetes versus no diabetes or diabetic subtype) are subject to the influence of differing baseline characteristics, and diabetes is being used as a marker to represent these differing characteristics. Although diabetics had similar baseline characteristics between treatment groups (prasugrel versus clopidogrel) and the size of this subgroup was robust ($n=3146$), randomization was not stratified by diabetic status, and the possibility of an unidentified imbalance between treatment groups exists. The results are strengthened by biological plausibility, a gradient of effect with disease severity, and consistency with previous data for intensive platelet inhibition in patients with DM. We did not measure hemoglobin A_{1c} or have another measure of DM severity or its medical control to better characterize the DM subgroup, although it would be expected that misidentification would be unusual and would only serve to bias the results toward the null. Finally, the absence of a large-scale platelet function study in subjects with DM in TRITON-TIMI 38 does not allow elucidation of the direct relationships between prasugrel, clopidogrel, platelet function, and outcomes. However, our study shows that an agent that achieves greater antiplatelet effect on a population basis had a larger effect on outcomes among subjects with than without DM.

Conclusions

In this analysis from TRITON-TIMI 38, we have demonstrated that DM has an independent adverse association with ischemic outcomes in subjects undergoing PCI for ACS, including a gradient from no DM to DM without insulin therapy to DM with insulin therapy. We have also shown that compared with standard clopidogrel therapy, intensive oral platelet inhibition with prasugrel resulted in greater benefit in reducing ischemic events and improving net outcomes among subjects with DM than in those without DM. These data demonstrate that intensive oral antiplatelet therapy is of particular benefit to patients with DM with ACS and planned PCI, and diabetes status should be considered when therapeutic options are weighed.

Disclosures

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CLINICAL PERSPECTIVE

Patients with diabetes mellitus (DM) are at high risk for recurrent cardiovascular events after acute coronary syndrome, in part because of increased platelet reactivity. Patients with DM have also been reported to be more likely to have a poor antiplatelet response to clopidogrel. The Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38) demonstrated an overall 19% reduction in ischemic events with more intensive antiplatelet therapy with prasugrel compared with clopidogrel, but with more bleeding. Of 13/608 subjects, 3146 had a history of DM. We observed that, despite modern therapy including coronary intervention, DM had an independent adverse effect on clinical outcomes. Moreover, we found that prasugrel was especially efficacious in patients with DM. Although key ischemic end points including the primary end point were significantly reduced among both patients with and without DM, greater absolute and relative reductions were seen in favor of prasugrel among subjects with DM, driven by a 5% absolute and 40% relative reduction in MI. No difference in TIMI major bleeding was observed in patients with DM, whereas a significant increase was observed in patients without DM. Combining safety and efficacy, prasugrel showed a net clinical benefit that was greater for patients with DM (26%) than without DM (8%). These data have implications for the potential use of prasugrel but also in a broader sense underscore the importance of intensive antiplatelet therapy for the growing population of patients with DM and acute coronary syndrome.